A Genetic Analysis of Two Strains of *Plasmodium chabaudi adami* that Differ in Growth and Pathogenicity

Naomi Jane Gadsby

Thesis Submitted for the Degree of Doctor of Philosophy
The University of Edinburgh
November 2008

Abstract

Malaria is still a significant public health problem in the Tropics, with an estimated 200 million cases a year and more than 1 million deaths, mostly in young children in sub-Saharan Africa. *Plasmodium falciparum* is the parasite responsible for the majority of the morbidity and mortality due to malaria. We know from the historical use of malaria to treat neurosyphilis that there were several different strains of *P. falciparum*, some of which were more pathogenic and had higher multiplication rates than others. High multiplication rates of *P. falciparum* isolates have been associated with severe disease in Thailand, but not in Kenya or Mali. In determining what differences exist between fast- and slow-growing malaria parasites, and understanding their relationship with clinical outcome, we may discover a way of targeting those parasites that cause most disease.

This thesis describes a genetic analysis of the determinants of growth and pathogenicity in the rodent malaria parasite, *Plasmodium chabaudi*. The use of rodent malaria parasite strains for genetic analysis has several experimental, ethical and financial advantages over the use of human malaria parasites. In addition, rodent malaria parasite strains also vary significantly in their growth and pathogenicity, making them excellent candidates for a genetic analysis of these characteristics. The first section of this thesis is concerned with the characterisation of the growth, pathogenicity and transmissibility of two strains, DS and DK, of the rodent malaria parasite *P. c. adami*. The DS strain is fast-growing, pathogenic, non-selective in its invasion of red blood cells and a poor transmitter to mosquitoes. The DK strain is slow-growing, non-pathogenic, selective in its invasion of red blood cells and a good transmitter to mosquitoes. In the second section of this thesis is a detailed study of the growth characteristics of DS and DK in mixed infections, relative to their growth in single infections. Both sections provide information relevant for the main objective of this thesis, but also contribute to the body of work on pathogenicity and transmissibility, and pathogenicity and strain behaviour in mixed infections, which has been carried out in rodent malaria parasites to-date.

The third section of the thesis contains the results of a genetic analysis of the difference in growth between *P. c. adami* strains DS and DK, using the Linkage Group Selection (LGS) technique. On several occasions, DS and DK were crossed in the mosquito vector and, following selection for fast growth in mice, the cross progeny were initially screened with genome-wide, quantitative AFLP markers. Markers specific to the slow-growing parent DK which were greatly reduced in intensity after selection were found on *P. chabaudi* chromosomes 6, 7 and 9. This result suggests that the difference in growth between the two strains is determined by multiple genetic loci. The selection on chromosomes 7 and 9 was then looked at in greater detail, using SNP-based markers quantified by Pyrosequencing™. It was found, consistently, that a region at one end of DS chromosome 9 was inherited as a single, non-recombining unit in cross progeny selected for fast growth. As this was the region most strongly selected against, it suggests that a gene (or genes) in this region has a major role in the determination of growth characteristics, and therefore pathogenicity, in *P. c. adami*. Narrowing down this region further, in order to identify the candidate gene(s), remains a key future objective.

Declaration of Contributions

I hereby declare that I alone have composed this thesis and that, excluding the contributions detailed below, the work and the opinions expressed within this thesis are my own.

The objectives of this project were conceived with the help of my principle supervisor Professor Richard Carter. Professor Richard Carter was also responsible for developing the Linkage Group Selection technique prior to my joining the laboratory and for contributing scientific guidance throughout this project.

Dr Sandra Cheesman and Dr Sittiporn Pattaradilokrat both contributed their knowledge of pyrosequencing and proportional AFLP techniques, respectively.

Mr Les Steven contributed to the monitoring of experimental animals and carried out the maintenance of the mosquitoes used in the project.

Two University of Edinburgh undergraduate students, Miss Ailie Robinson and Mr Richard Lawrence, were involved in some of the data collection carried out for experiments in Chapters 2 and 3.

Finally, Dr Nick Colegrave and Dr Katherine Baldock were consulted for statistical advice for the analysis of mosquito transmission data in Chapter 2.

I hereby declare that this work has not been submitted for any other degree or professional qualification.

Naomi Gadsby

November 2008

Acknowledgements

I would like to acknowledge the help and guidance of Professor Richard Carter who has patiently supervised my PhD studies in his laboratory. Similarly, the expertise of other members of the laboratory past and present: Dr Sandra Cheesman, Dr Richard Culleton, Dr Sittiporn Pattaradilokrat, Dr Elaine O'Mahoney, Dr Paul Hunt, Dr Alison Creasey, Mrs Kathryn Degnan and Mr Les Steven, has been invaluable. I am also grateful to Mrs Bette Boyle, Mr Ronnie Mooney, Mr Richard Fawcett, Mrs Sheena Boath and Mr Ray Mclnnes, who all provided excellent technical assistance. Furthermore, I would like to thank my second supervisor Dr Alex Rowe for her interest and encouragement.

Having been born on the 20th August 1982, 85 years to the day that Ronald Ross discovered that malaria was transmitted by the mosquito and 11 years to the day that David Walliker and Richard Carter published the first genetic cross between two malaria parasites, I feel that it was probably fate, rather than free will, which led me to undertake a PhD on malaria parasite genetics. However, I have been very lucky to have had the chance to work in this field and for that I am very grateful to my supervisors and to the Wellcome Trust for awarding me a place on their PhD programme at the University of Edinburgh.

Similarly, I have been very lucky to meet along the way many friends who have made my time as a PhD student in Edinburgh such an enjoyable one, especially thanks to Jo Konkel, John Grainger, Vicki Barclay, Lasani Wijetunge, Will Chadwick, Lola Oyebode, Matt Lyst and Nick Wilkie.

To my parents and my sister, for their continuing support and enthusiasm for my various endeavours, thank you again.

Finally, to my partner Manousos Syngellakis, who with unfailing understanding, put up with much neglect for the greater scientific good during the course of my PhD, and yet was always there for me, thank you very much.

Table of Contents

Page Number

List of Figures

Page

Number

Chapter 1: Introduction

Chapter 2: Growth, Pathogenicity and Transmissibility of *P. c. adami* DS and DK

Chapter 3: *P. c. adami* DS and DK Growth in Mixed Infections

Chapter 4: LGS Analysis of the Difference in Growth of *P. c. adami* Strains DS and DK: Part 1.

Chapter 5: LGS Analysis of the Difference in Growth of *P. c. adami* Strains DS and DK: Part 2.

Chapter 6: Investigation of the Stage of the Lifecycle in which Selection acts on Cross Progeny

Appendix I: Pyrosequencing Markers

Appendix II: Clonal Cross Progeny Marker Inheritance

List of Tables

Page Number

Abbreviations

AFLP	Amplified Fragment Length Polymorphism
AMA-1	Apical membrane antigen 1
ANOVA	Analysis of variance
aroC	Gene encoding chorismate synthase
ATP	Adenosine triphosphate
bir	Gene encoding *P. berghei* interspersed repeats
BLAST	Basic Local Alignment Search Tool
cir	Gene encoding *P. chabaudi* interspersed repeats
cM	centiMorgan
CSP	Circumsporozoite protein
DBL	Duffy binding-like
DDT	Dichloro-Diphenyl-Trichloroethane
DHFR	Dihydrofolate reductase
dhodh	Gene encoding dihydroorotate dehydrogenase
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
etramp	Gene encoding *P. falciparum* early transcribed membrane protein
GDH	NADP-dependent glutamate dehydrogenase
GDP	Gross Domestic Product
GPI	Glucose phosphate isomerase
HRP-3	Histidine-rich protein 3
HSPG	Heparan sulphate proteoglycans
i.p.	Intra-peritoneal
kir	Gene encoding *P. knowlesi* interspersed repeats
LDH	Lactate dehydrogenase
LGS	Linkage Group Selection
LSA-1 and 3	Liver stage antigens 1 and 3
ml	Millilitre
MSP-1, 2 and 7	Merozoite surface proteins 1, 2 and 7
NCBI	National Centre for Biotechnology Information (US)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1

PfHRP2	*Plasmodium falciparum* histidine-rich protein 2
PfRH1	*Plasmodium falciparum* reticulocyte binding-like homologue 1
pir	Gene encoding *Plasmodium* interspersed repeats
PMR	Parasite multiplication rate
PPi	Pyrophosphate/inorganic phosphate
pRBC	Parasitised red blood cell
PSQ	Pyrosequencing
QTL	Quantitative trait locus
RBC	Red blood cell
RI	Relative Intensity
rif	Gene encoding *P. falciparum* repetitive interspersed family
RMP	Rodent malaria parasite
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SALSA	Sporozoite and liver-stage antigen
SEM	Standard error of the mean
SI	Selectivity Index
SNP	Single nucleotide polymorphism
SPECT	Sporozoite microneme protein essential for cell transversal
STARP	Sporozoite threonine and asparagine-rich protein
TRAP	Thrombospondin-related anonymous protein
UV	Ultraviolet
vir	Gene encoding *P. vivax* interspersed repeats
yir	Gene encoding *P. yoelii* interspersed repeats
WHO	World Health Organisation
µM	microMolar
µl	microlitre
6-PGD	6-phosphogluconate dehydrogenase

Chapter 1: Introduction

1.1 The Global Impact of Malaria: Past and Present

In Shakespeare's The Tempest, Caliban, the wild, tormented slave of Prospero curses his master;

> "All the infections that the sun sucks up
> From bogs, fens, flats, on Prosper fall and make him
> By inch-meal a disease."
>
> *The Tempest, Act II Scene II*

The disease he is wishing on Prospero is none other than malaria, which festered in the late summer and autumn in the "unwholesome fen[s]" of England. Later on, Caliban, shaking with fear on seeing Stephano approach, appears to "hath got, as I take it, an ague" which Stephano then tries to cure with alcohol; "If all the wine in my bottle will recover him, I will help his ague".

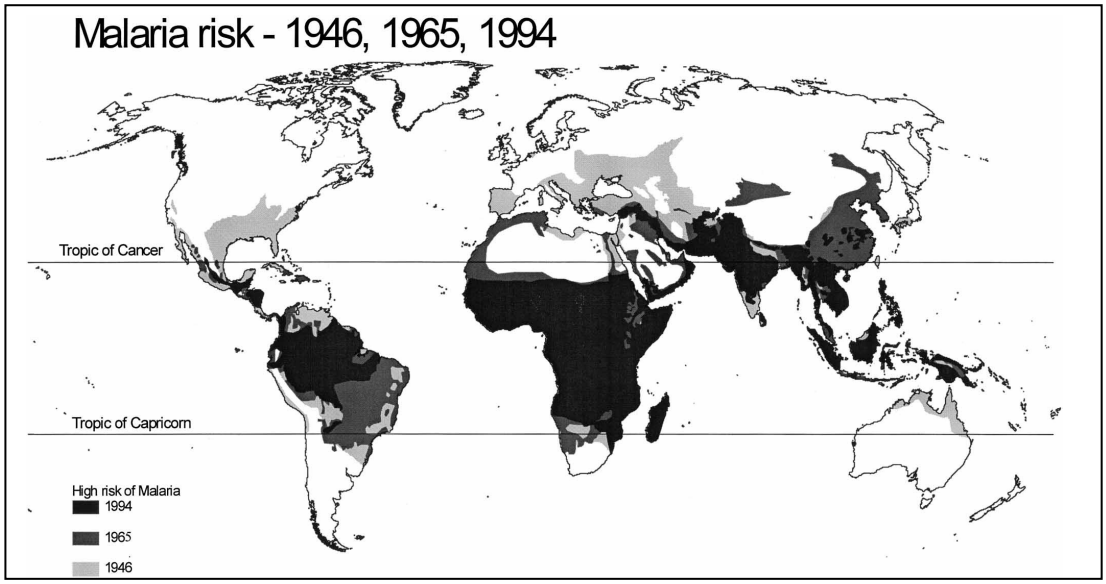
Today, however, treatments of a more pharmaceutical nature are at hand and "the ague" has thankfully been banished from England. However, malaria has had a dramatic effect on human health for hundreds, if not tens of thousands, of years. Traces of the human malaria parasite *Plasmodium falciparum* have even been found in the mummified remains of individuals who lived in ancient Egypt between 3200BC and 1304BC (Miller et al. 1994). Malaria once spread widely across the globe, and at a time when the world's population was less prosperous and well-nourished than today, its effects were devastating. This was particularly so in areas of moderate transmission where the population were often exposed to malaria, but at a level insufficient to build up any protective immunity (Carter and Mendis 2002). The negative effect of malaria on human health was so strong that many populations

across the globe have been left with a legacy of blood disorders whose protective effects from the scourge of malaria have ensured their persistence (Min-Oo and Gros 2005). Due to the implementation of drug treatment, widespread mosquito vector eradication campaigns and the improvements in general health and housing that came from increased prosperity, this burden was reduced dramatically in the temperate regions of the world during the 20th century (Carter and Mendis 2002) (Figure 1.1).

To date, there have been three Nobel Prizes awarded for land-mark discoveries made in malaria research. In 1907, Charles Laveran was awarded the Nobel Prize for his discovery of the causative agent of malaria in 1880, by observing what later came to be recognised as gametocyte exflagellation, in the blood of a French soldier fighting in Algeria. Ronald Ross was awarded the Nobel Prize in 1902 for his discovery, in India in 1897, of oocysts in *Anopheles* mosquitoes which had been previously fed on malaria-infected blood, thus proving the nature of malarial parasite transmission. Finally, the third Nobel Prize went to Paul Miller in 1948 for his discovery of the malaria vector insecticide DDT, which went on to be the main-stay of the WHO malaria eradication programme. Despite over 100 years of research into malaria, the world today is desperately in need of a new discovery and a fourth malaria Nobel laureate, because for many of the world's population, Shakespeare's "ague" is still a reality.

Today, malaria remains a significant public health and economic problem in tropical and sub-tropical regions (Sachs and Malaney 2002). About 100 countries or territories are currently considered malarious, almost half of which are in sub-

Figure 1.1
The changing global distribution of malaria risk over the 20th century (image reproduced from (Gallup and Sachs 2001).



Saharan Africa, meaning that more than 2.4 billion people are likely to be at risk (WHO 2000). An estimated 300-500 million cases worldwide occur annually, with an estimated 1.1-2.7 million deaths, the majority of which are due to *Plasmodium falciparum* infection in young children in sub-Saharan Africa (WHO 2000). However, a reliable estimate of the annual prevalence of and mortality due to malaria is difficult to obtain because of incomplete case-reporting and fluctuating malaria transmission patterns (Snow et al. 2005; Carter and Mendis 2006).

In areas of high transmission, clinical immunity to malarial disease is built up over years of repeated infections so that an adult may still carry parasites in their blood and yet show no clinical symptoms. However, this is at the price of enormous infant mortality. Most deaths occur in children less than 5 years old because this age group have not yet been able to acquire such protection. High rates of childhood mortality lead to high birth rates and increased population size, putting more strain on limited resources (Sachs and Malaney 2002). In those children that survive, the high rate of symptomatic infection in childhood is a large economic burden. Drugs and healthcare, if available, are expensive relative to a typical family's income and, additionally, looking after a sick child reduces the time a parent can spend at work. Given that most families are likely to be on low incomes, and that seasonal transmission often coincides with harvest time, malaria certainly exacerbates an already financially precarious situation.

At a national level, the presence of malaria puts a large strain on already over-stretched and under-funded public health infrastructures. It is estimated that in some

countries with heavy malaria burdens, it accounts for up to 40% of public health expenditure (WHO 2001). According to a report released by the WHO to mark the beginning of the African Roll Back Malaria Summit in 2000, Africa's GDP would have been up to 32% higher, or $100 billion greater, in the year 2000 if malaria had been eradicated in 1965. Between 1965 and 1990, GDP growth was 5 times higher in non-malarious countries compared to malarious countries (Gallup and Sachs 2001). It is in these malarious countries that economic development and investment is most needed and is also where it is least likely to occur, given its malarious nature (Gallup and Sachs 2001; Sachs and Malaney 2002). Added to the impact of other communicable diseases such as HIV and tuberculosis, malaria is undoubtedly a large contributor to the vicious cycle of disease and poverty which has its biggest impact in the countries of sub-Saharan Africa.

1.2. The *Plasmodium* Lifecycle

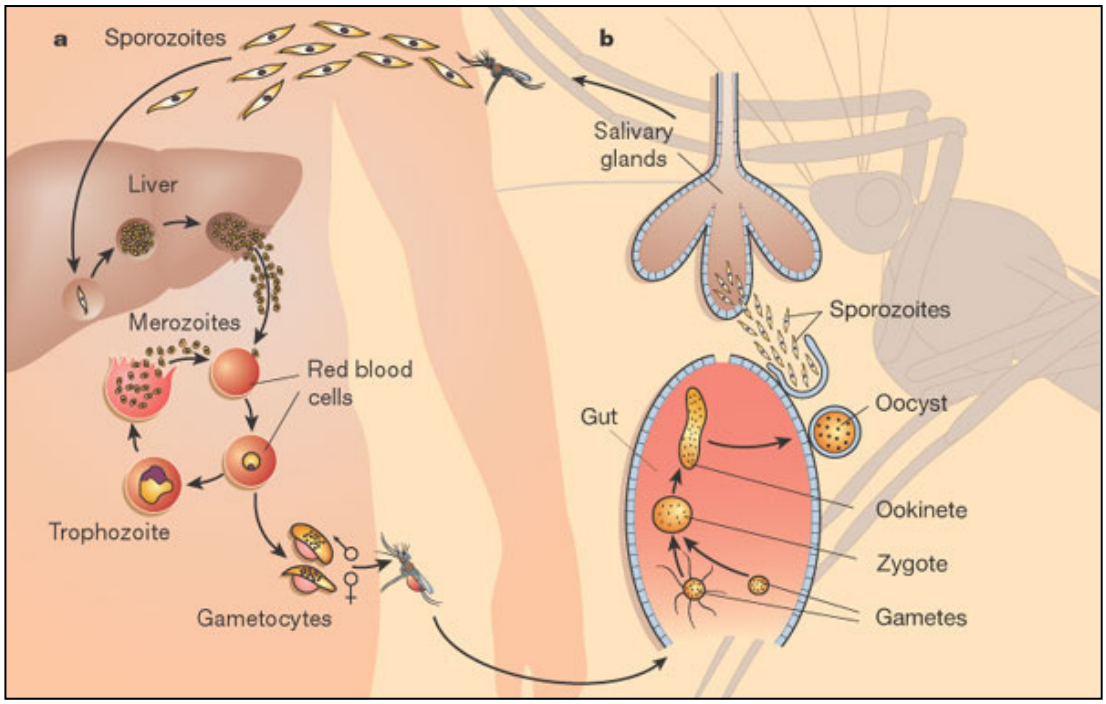
Plasmodia are members of the Apicomplexa phylum of unicellular, intracellular parasites. They are transmitted between hosts by members of the Culicidae family such as *Anopheles* mosquitoes. Four *Plasmodium* species regularly infect humans; *P. falciparum, P. vivax, P. ovale* and *P. malariae*. Today, the causative agent of the deaths and morbidity due to malaria is usually *P. falciparum*, the most pathogenic of the four *Plasmodium* species that infect humans. However, in the past, *P. vivax* and to a lesser extent *P. malariae* and *P. ovale,* have all contributed to the large global burden of malaria morbidity (Carter and Mendis 2002). In addition, a fifth *Plasmodium* species that normally infects long-tailed and pig-tailed macaques in south east Asia, *P. knowlesi*, has been recently found to cause zoonotic infections of

humans (Cox-Singh et al. 2008). Natural human-human transmission of *P. knowlesi* has not been observed, apparently due to the habitat preference of the mosquito vector, thereby reducing the potential of this fifth species to become a fully established parasite of humans at the present time.

The lifecycle of *P. falciparum* is shown in Figure 1.2 (Wirth 2002). Haploid sporozoites are inoculated by the female Anopheline mosquito into the host when she lands on the skin in pursuit of a blood meal. The motile sporozoites then proceed through the blood to the liver; this stage of malarial infection is asymptomatic. The sporozoites must migrate through dermal cells in the skin and the endothelial cells of blood vessels before reaching the liver. In a recent imaging investigation in an animal model, many sporozoites in fact remained in the skin, whilst others became trapped in lymphatic vessels (Amino et al. 2006). Therefore, many sporozoites may never actually reach the liver and may interact with the immune system in the periphery.

Upon reaching the liver, sporozoites must also traverse the liver sinusoidal barrier before invasion of hepatocytes, and it is possible that this occurs through the resident liver macrophages, Kupffer cells (Pradel and Frevert 2001). It is clear that migration through several hepatocytes may also occur before one is productively invaded. Exactly how the parasite determines when to invade a cell and when to migrate through it is not entirely understood, although it might be due to the level of highly sulphated heparan sulphate proteoglycans (HSPGs) on hepatocytes and their

Figure 1.2
The lifecycle of *Plasmodium falciparum* in the human host and female *Anopheline* mosquito (image reproduced from (Wirth 2002).



interaction with the parasite circumsporozoite protein (CSP) (reviewed in (Prudencio and Mota 2007). Sporozoite proteins including CSP, thrombospondin-related anonymous protein (TRAP), sporozoite threonine and asparagine-rich protein (STARP), liver-stage antigens 1 and 3 (LSA-1 and LSA-3), sporozoite and liver-stage antigen (SALSA), and sporozoite microneme protein essential for cell transversal (SPECT), are all thought to be involved in migration through and invasion of liver cells, but a precise mechanism has not yet been established (reviewed in (Garcia et al. 2006).

Once a sporozoite has invaded a hepatocyte, it undergoes a round of asexual reproduction; a single sporozoite produces a single hepatic schizont. Within two days for rodent malaria parasites, and six to seven days for species that infect primates, tens of thousands of haploid merozoites are released from hepatic schizonts (White 1998; Frevert 2004). This first generation of merozoites are thought to be released directly from the liver into the blood stream (Figure 1.2). However recent work, again in an animal model, suggests that they may in fact be packaged into membrane-bound "merosomes" which travel to the lung capillary beds where the merozoite cargo is finally released (Baer et al. 2007). In *P. vivax* and *P. ovale* infections, sporozoites entering hepatocytes can develop into hypnozoites which can lie dormant for months or even years, and once re-activated, produce merozoites leading to a relapse of infection (Covell and Nicol 1951; Cogswell 1992).

Malarial merozoites are very small, only 1.5-2.5μm long and 1-2μm wide, and they rapidly invade erythrocytes (Barnwell and Galinski 1998). The invasion process

takes only 30-60 seconds and there are 3 main phases: (1) the initial attachment of the merozoite to the erythrocyte surface, (2) reorientation of the merozoite so the apical pole is positioned on the erythrocyte surface and (3) tight junction formation and entry of the merozoite into the host cell, coincident with the formation of the parasitophorous vacuole (Barnwell and Galinski 1998). In *P. falciparum*, the initial attachment of the merozoite to the erythrocyte surface is possibly due to the interaction of host red blood cells with the parasite merozoite surface proteins, particularly the MSP-1 protein which is proteolytically cleaved into a 19kDa fragment (Gaur et al. 2004). Similarly, the parasite apical membrane antigen 1 (AMA-1) protein may be needed in apical reorientation, which is the next phase of invasion.

The formation of the tight junction between the merozoite and erythrocyte membranes is an irreversible step which leads to invasion, and there are many *P. falciparum* proteins from the Duffy Binding-Like (DBL) superfamily and the Reticulocyte Binding-Like Homologue (PfRh) family which have been implicated in this process (reviewed by (Gaur et al. 2004). However, very few host receptors have been identified for *P. falciparum* erythrocyte invasion and it is clear that different parasite isolates can invade erythrocytes in sialic acid-dependent and independent processes, and can even switch between these pathways when subjected to a selection pressure (Gaur et al. 2004). The apparent redundancy of *P. falciparum* invasion pathways and the large number of parasite and host proteins which might be responsible for invasion under different circumstances, makes it difficult to elucidate exactly how this parasite enters a red blood cell. However, not all red blood cells are

equally likely to be invaded; only approximately 40% of circulating erythrocytes were susceptible to *P. falciparum* invasion in a Thai study (Simpson et al. 1999). This compares with 13% of circulating erythrocytes which were susceptible to invasion by *P. vivax* in the same study, which is consistent with the known preference of *P. vivax* for reticulocyte or young red blood cell invasion (Simpson et al. 1999).

Once inside the red blood cell, *P. falciparum* develops into a ring stage/early trophozoite surrounded by a parasitophorous vacuole and remains at this stage for around 12 hours (Figure 1.2) (Bozdech et al. 2003). It then develops into a highly metabolically active late stage trophozoite and remains at this stage until around 26 hours post-invasion. Finally, trophozoites develop into schizonts and asexual replication occurs. Merozoites are formed within the schizont and are released when the red blood cell bursts at around 48 hours post-invasion. This cycle of red blood cell invasion and rupture takes around 48 hours in *P. falciparum*, *P. vivax* and *P. ovale*, but around 72 hours in *P. malariae*. However, *in vitro*, different strains of *P. falciparum* have been found to vary between 44-50 hours in their intra-erythrocytic cycle time (Reilly et al. 2007).

Each schizont, generated by a single merozoite, produces an average of around 16 haploid merozoites (range 8-24) although again, this may vary between *P. falciparum* strains *in vitro* (Reilly et al. 2007). The actual in-host parasite multiplication rate per 48 hour period appears to be much lower, and has been measured as approximately 8-fold per cycle, in the initial 7 days of infection with *P.*

falciparum in non-immune individuals (Simpson et al. 2002). After this time, the immune response will usually begin to limit further parasite population expansion to 1-fold or less per cycle (Simpson et al. 2002).

The time taken from the inoculation of sporozoites to the appearance of clinical symptoms (the incubation period) in malaria infections can typically take 7-14 days (Covell and Nicol 1951). *P. falciparum* parasites are usually detected in the blood by the 11^{th} day of infection, with symptoms occurring by the 13^{th} day (White 1998). Symptoms of *P. falciparum* infection include the classic malaria paroxysm - chills, fever, and sweats, and more rarely, severe anaemia, cerebral malaria, lactic acidosis and respiratory distress.

As the mature trophozoites and schizonts of *P. falciparum* sequester in capillaries in major organs such as the lungs and the brain, only immature trophozoites (ring stages) are seen in the peripheral blood. Therefore, as parasites leave the peripheral circulation during the 48 hour intra-erythrocytic cycle and then reappear after schizogony, daily parasite density measurements can often form a sine wave, oscillating around a steadily increasing mean parasite density (Simpson et al. 2002). However, *P. falciparum* populations are not always synchronous and, therefore, different sub-populations at different stages of development may sequester at different times (Simpson et al. 2002). The appearance of mature forms like schizonts in the circulation is thought to be a sign of a very high parasite burden and has a poor prognosis for the patient (Covell and Nicol 1951; Dondorp et al. 2005).

In the blood stage of the lifecycle, although the majority of invading merozoites will go on to develop into schizonts and continue the asexual cycle, a small proportion (usually less than 10%, and subject to change over time) will go on to develop into haploid male or female gametocytes, the sexual stage ready for transmission to the mosquito (Figure 1.2) (Smalley et al. 1981). All the merozoites in a single schizont appear to be committed to develop into either asexual parasites or gametocytes, but not a mixture of the two (Bruce et al. 1990). However, how they are developmentally programmed in this way is not yet understood. In order for transmission to occur, an infected host must be bitten by a female mosquito and both male and female gametocytes must be taken up during the blood meal.

Female and male gametocytes enter the insect gut where their exflagellation is stimulated and they develop into macro- and micro-gametes respectively. The fusion of a microgamete with a macrogamete leads to fertilisation, briefly forming a diploid zygote in which sexual reproduction occurs. This then develops into a motile ookinete which traverses the gut wall, embeds itself on the outside of the mosquito gut and develops into an oocyst (Figure 1.2). Each oocyst, generated by the fusion of a single male and female gamete, contains 4 meiotic products, each of which is replicated asexually. Asexual reproduction produces haploid sporozoites which remain inside the maturing oocyst for 4-15 days; the oocyst grows to a sphere of between 6-50μm in diameter which projects out into the haemocoel cavity (Garnham 1966). An estimated 10,000 sporozoites can be released from an oocyst; these travel from the gut into the whole body of the mosquito, ultimately penetrating the salivary glands (Garnham 1966). Here the sporozoites remain, ready to be inoculated into the

next host and begin a new cycle of infection when the female mosquito takes her next blood meal.

Therefore, three organisms are involved in malarial infection; parasite, host and vector. In order to reduce the burden of malaria in the Tropics, interventions can be designed to target several stages of this varied parasite lifecycle. For example, the parasite can be targeted in the host either directly with drugs, or potentially indirectly with vaccines or therapeutic antibodies to improve the protective host immune response. Similarly, strategies that reduce vector contact with the host, such as bed-nets, can be employed to reduce the number of new infections. In order to apply these strategies and to develop new ones, we need to know more about the biology of the malaria parasite and its interaction with the host. This thesis contributes to the body of research in this area.

1.3. Factors Determining the Severity of *P. falciparum* Malaria Infections

The repeated rounds of parasite asexual reproduction in the blood are responsible for the clinical symptoms of malaria. On a simple level, many red blood cells are destroyed and this leads to anaemia. However, during the erythrocytic cycle, the parasite is involved in a complex interaction with both the innate and acquired immune response of the host, the implications of which are not yet fully understood. The inflammatory reaction to parasite-derived products following schizont rupture is thought to produce the paroxysms which generally occur (although irregular, intermediate fevers may also be seen) every 2nd day for the tertian malarias *P. falciparum*, *P. vivax* and *P. ovale*, and every 3rd day for the quartan malaria *P.*

malariae. *P. falciparum* mature trophozoites and schizonts sequester in capillary beds in major organs such as the brain and lungs, and this may lead to a reduction in tissue perfusion or localised immune-mediated effects on endothelial cells. In the brain, parasite sequestration is thought to lead to cerebral malaria, but the exact mechanism is unknown.

Clinical cases of *P. falciparum* malaria can be broadly categorised into "uncomplicated" or "severe" according to WHO criteria (Warrell 1990). The severe forms of *P. falciparum* malaria manifest differently in different populations. In areas of low transmission and therefore low acquired immunity, such as Thailand, severe disease is found in individuals of all ages (Dondorp et al. 2005). In areas of high transmission and high levels of acquired immunity to clinical disease, such as sub-Saharan Africa, severe disease is mainly seen in children under 5 years old and in pregnant women. In children, severe anaemia, hypoglycemia and convulsions are more common, whereas adults are more likely to develop jaundice, renal failure or acute pulmonary oedema (White 1998).

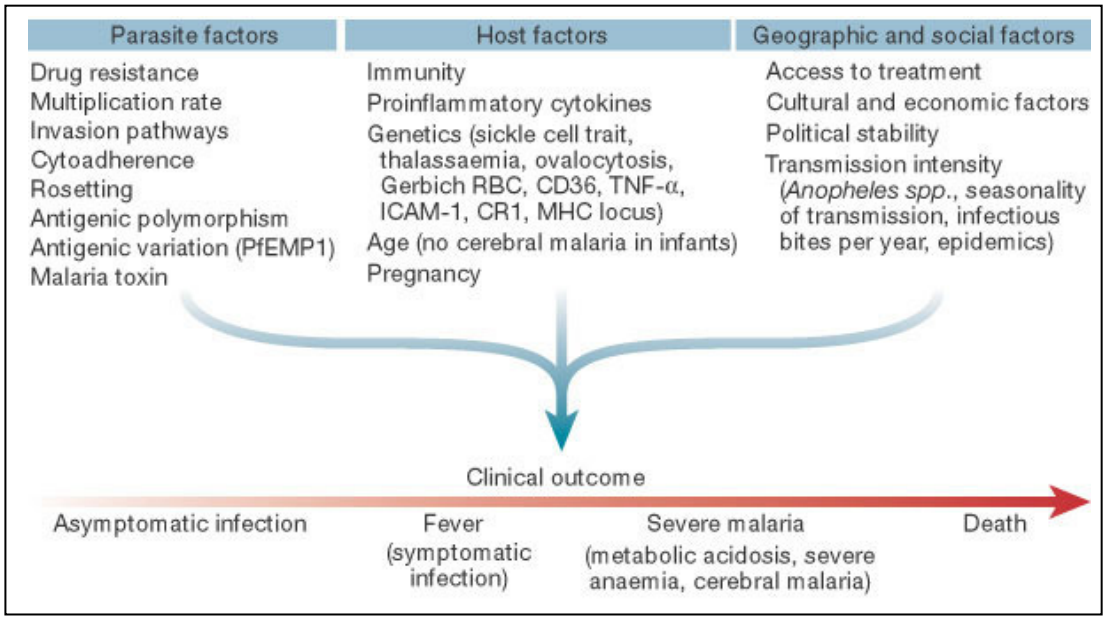
Although most individuals will make a full recovery from severe disease, it can be fatal in around 10% of cases or, in the case of cerebral malaria specifically, lead to permanent neurological impairment (Brewster et al. 1990). A recent, large study at several centres across Asia indicates that in non-immune individuals from low transmission areas, mortality from severe malaria increases with age from 6.1% in children less than 10 years old, to 36.5% in adults older than 50 (Dondorp et al. 2008). In the UK there are around 1500-2000 imported malaria cases annually, and

despite good healthcare infrastructure, there is still a mortality rate of around 1% (Lalloo et al. 2007).

Why a small proportion of people infected with *P. falciparum* go on to develop a severe or even fatal disease, whilst the majority show mild symptoms or even none at all, is still a key question in malaria research (Marsh 1992). The large spectrum of disease severity, ranging from mild fever to death, could be determined by a combination of many parasite, host and environmental factors, as illustrated in Figure 1.3 (Miller et al. 2002). Therefore it is possible that a death from malaria is the unfortunate result of an infection with the "wrong" parasite, in the "wrong" host, at the "wrong" time.

However, some parasite or host properties have been linked to differences in disease severity in particular populations. For example; rosetting (the ability of a parasitised red blood cell to surround itself with two or more uninfected red blood cells) (Rowe et al. 1995; Rowe et al. 2002); platelet-mediated clumping of parasitised red blood cells (Pain et al. 2001); parasite multiplication rate and selectivity of red blood cell invasion (Chotivanich et al. 2000); the expression of particular subsets of parasite *var* genes encoding the variant surface protein and cytoadhesion ligand PfEMP1 (Kaestli et al. 2006; Kyriacou et al. 2006); and host red blood cell characteristics such as the haemoglobin E trait (heterozygotes with HbE/HbA) (Hutagalung et al. 1999) and blood group O (Rowe et al. 2007), have all been implicated.

Figure 1.3
The spectrum of clinical disease caused by *Plasmodium falciparum* and factors which may influence pathogenesis (image reproduced from (Miller et al. 2002).



Similarly, environmental factors appear to have a strong influence on malaria incidence and severity. The number of clinical episodes of *P. falciparum* malaria over two years, in children from different Ghanaian villages in a high transmission area, was found to be affected by a number of socio-economic factors (Kreuels et al. 2008). Additionally, incidence was strongly linked to the proximity of children's households to the fringe of the forest, indicating possible differential vector contact, even within a small location in an area of high transmission. Temporal-spatial clustering of cases of severe malaria was found in coastal Kenya over a three-year period (Snow et al. 1993); this could be explained by a number of factors including localised differences in the vector population or the existence of particular parasites causing more severe disease than others.

Several genetic studies have been undertaken in different countries to look for associations of particular alleles of genes involved in cell invasion and adhesion, or particular microsatellite haplotypes, with severe disease in *P. falciparum* infections (Engelbrecht et al. 1995; Robert et al. 1996; Kun et al. 1998; Ariey et al. 2001; Ferreira et al. 2002; Ranjit et al. 2005; Martha et al. 2007; Toure et al. 2007). In some studies, as in Senegal and Vietnam respectively, no association was found between particular genotypes and severe disease (Robert et al. 1996; Ferreira et al. 2002). In other locations, different associations with alleles of the same genes have been found; in India, a MAD20 allele of *msp1* and a 3D7 allele of *msp2* were associated with severe malaria (Ranjit et al. 2005), however, in Papua New Guinea, the FC27 allele of *msp2* was associated with severe disease (Engelbrecht et al. 1995) whereas the *msp1* B-K1 allele and the varD allele were associated with severe

disease in French Guiana (Ariey et al. 2001). Even within the same country, the association of the K1 allele of *msp1* found in one study in Gabon to be associated with severe disease (Kun et al. 1998) was not found in a later study (Toure et al. 2007). Some of these studies are small and there is no consensus between them, except to illustrate that where associations of particular gene alleles and disease severity are found, they can be expected to vary geographically.

Ascertaining exactly what contribution different host, parasite and environmental factors make to disease outcome is crucial for understanding the basic biology of the malaria parasite and its interaction with the host. As these factors may vary in different populations, such knowledge will be important for the development of intervention strategies which are appropriate for a particular region. More specifically, disentangling the parasite factors which might be at least partly responsible for severe disease would inform the development of new drugs and potential vaccines against malaria. With this in mind, it is of great interest whether particularly pathogenic "strains" or populations of *P. falciparum* are responsible for severe malarial disease in the field (Marsh 1992; Gupta et al. 1994).

1.4. *P. falciparum* Multiplication Rate and Association with Pathogenicity

Evidence that different isolates of the same species, *P. falciparum*, can have different characteristics comes from careful scientific observations made during the practice of inducing malaria infections in naïve individuals for the treatment of neurosyphilis in the first half of the 20th century (James et al. 1932; Boyd et al. 1936; Covell and

Nicol 1951; Jeffery 1966; Simpson et al. 2002). G. Covell and W. D. Nicol (1951) state that :

> "…within the species [*P. falciparum*] there exists a number of geographical races which, though not morphologically different, can be recognised as distinct by their clinical virulence, immunological reactions and other properties".

In particular, the now extinct European strains of *P. falciparum* from Italy and Sardinia appeared to cause more parasite recrudesces and fevers of longer duration, than those from India and Africa (James et al. 1932; Covell and Nicol 1951).

A retrospective data analysis of *P. falciparum* isolates used in the treatment of neurosyphilis in the United States found that although the parasite multiplication rate in the initial 7 days of infection (PMR) varied between individuals infected with the same strain, each strain had its own distinct PMR (Simpson et al. 2002). In a recent study of naturally acquired *P. falciparum* infections in Thailand, an area of low transmission and consequently low host immunity, isolates from patients with severe disease had high parasite multiplication rates. Isolates from Thai severe malaria cases multiplied three times as fast, and were less selective in their invasion of erythrocytes, than isolates from uncomplicated malaria cases (Simpson et al. 1999; Chotivanich et al. 2000). However, in Mali and Kenya, which are areas of high transmission with high levels of acquired immunity, overall multiplication rates of parasites were low compared to those in the Thailand study, and there was no significant difference in selectivity of invasion or multiplication rate, between isolates from severe and uncomplicated cases (Deans et al. 2006).

In the Thai population, patients with severe disease also had very high parasite burdens. Using plasma levels of PfHRP2 (a Histidine-Rich Protein released by the parasite) to determine total parasite numbers, there was a six-fold higher parasite burden in patients with severe compared to uncomplicated malaria, and this was two-fold higher still in fatal cases (Dondorp et al. 2005). This reflects much earlier observations of *P. falciparum* infections in Malaysia that demonstrated that very high parasite burdens were indicative of a poor prognosis, despite immediate drug treatment (Field and Niven 1937). These findings reveal an association between high parasite multiplication rates, leading to high parasite burdens, and pathogenicity, over and above the effects of host differences, in individuals with low or absent acquired host immunity. The production of a large number of asexual parasites, which is apparently detrimental to the host, might be a parasite strategy to increase the chances that deleterious mutations are nullified by compensatory mutations, ensuring the production of functional transmission stages (Hastings et al. 2004).

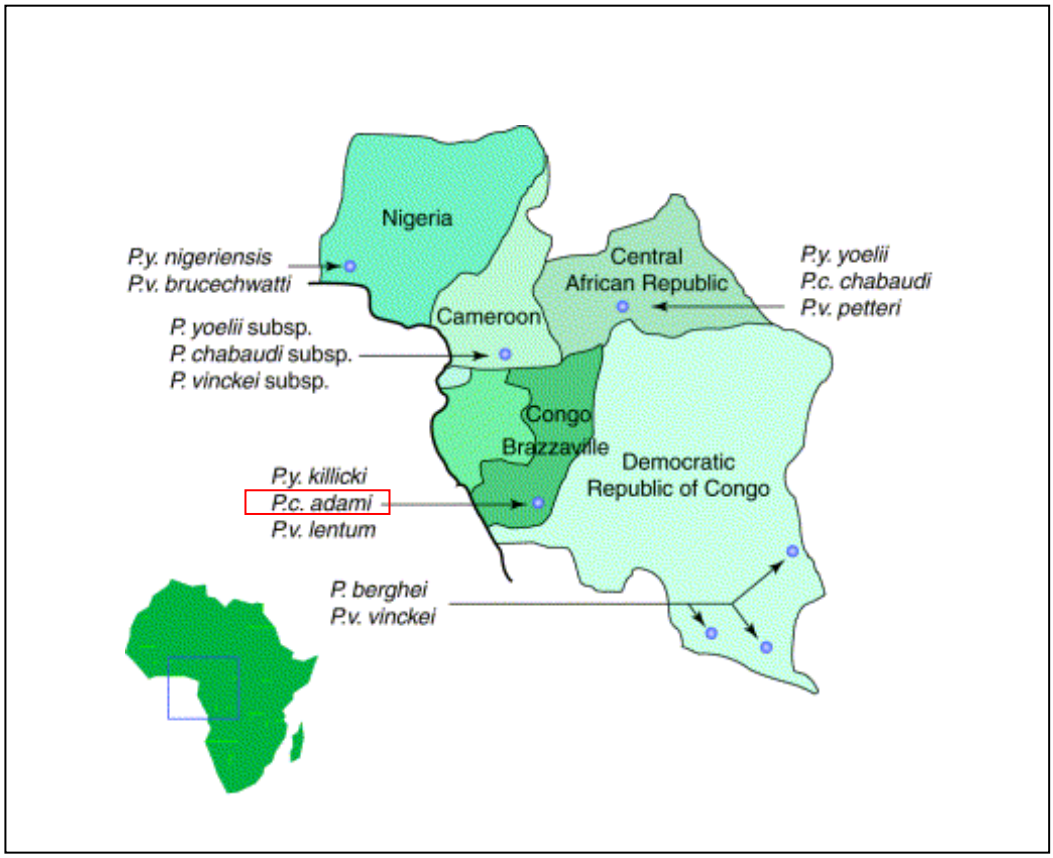
In sub-Saharan Africa, however, although children with severe malaria often have higher parasitaemias than those with uncomplicated infections, children with very high parasite burdens (hyperparasitaemia), but no sign of disease, are commonly found (e.g.(Deans et al. 2006). In the Malian study, parasite isolates from hyperparasitaemia cases actually had lower multiplication rates than those from severe or uncomplicated cases (Deans et al. 2006). Therefore the relationship between parasite multiplication rate, parasite burden and disease severity is not straight-forward, and is likely to be influenced by the different levels of immunity, or other factors, present in different geographical areas.

1.5. *P. chabaudi* Growth Properties and Association with Pathogenicity

A logical approach to dissect this issue would be to take malaria parasites of differing pathogenicity and compare their growth properties, using particular hosts and environments. However, aside from the historical work on malaria therapy for neurosyphilis, these kinds of studies cannot be carried out using *P. falciparum* in the human host. Therefore, the naturally occurring *Plasmodium* infections of rodents can be used to enable the identification of parasite specific factors important *in vivo*, through real-time, controlled experiments. *Plasmodium* species are known to infect a wide range of animals (Gilles 1993) and studies using rodent malaria parasites have provided many insights into *Plasmodium* biology, such as the mechanisms of drug resistance (Carlton et al. 2001).

Around 60 years ago a period of much interest in the naturally occurring malaria infections of African rodents began, because of their potential as a way of studying malaria parasite biology in mammalian hosts. The field began in 1943 with the discovery by the Belgians Dr Ignace Vincke and the Compte d'Ursel, of the presence of sporozoites in *Anopheline* mosquitoes which did not feed on humans, in a region of the Belgian Congo (now the Democratic Republic of Congo) (Garnham 1966). *P. berghei* was subsequently discovered in 1948 by Vincke in the blood of an African rodent, *Grammomys surdaster*, also from the Belgian Congo (Garnham 1966). In the following years, three other rodent malaria species, *P. chabaudi*, *P. yoelii* and *P. vinckei*, were isolated and the transmission and maintenance of these parasites was established in the laboratory (Figure 1.4) (Killick-Kendrick and Peters 1978).

Figure 1.4
Rodent malaria parasites were isolated from several locations in West Africa in the 1960s and 1970s (image adapted from (Carlton et al. 2001). *Plasmodium chabaudi adami*, the parasite subspecies which will be used in this study, is highlighted by the red box.



The rodent malaria parasite *P. chabaudi* was first described in 1965 in wild-caught thicket rats (*Thamnomys rutilans*) in the Central African Republic by a group of French scientists working in the field (Landau 1965) (Figure 1.4). They subsequently sent thicket rats from the Central African Republic and Congo (Brazzaville) to the Institute of Animal Genetics at the University of Edinburgh, from which several different strains of *P. chabaudi* (of two subspecies, *P. c. chabaudi* and *P. c. adami)*, were isolated (Carter and Walliker 1975, 1976). The large number of *P. chabaudi* strains at Edinburgh could be distinguished by differences in enzyme isoforms (isozymes) of the following: glucose phosphate isomerase (GPI), 6-phosphogluconate dehydrogenase (6-PGD), lactate dehydrogenase (LDH) and NADP-dependent glutamate dehydrogenase (GDH), as determined by starch gel electrophoresis (Carter 1978).

Rodent malaria parasite strains vary in their growth properties in the host and in their pathogenicity; and these two characteristics are often, but not always, associated (Fahey and Spitalny 1987; Mackinnon and Read 1999). Higher parasitaemias tend to lead to more rapid weight loss, anaemia and death (Mackinnon and Read 2004). Since infections with different parasite strains are carried out in the same strains of inbred laboratory mice, intrinsic genetic differences between parasites rather than hosts, is likely to determine the differences in growth. The parasite-derived differences in the growth properties of these strains could be due to several factors, e.g. variation in selectivity of red blood cell invasion, multiplication ability, and evasion or manipulation of the host immune response. Determining the mechanism and genetic basis for the differences in parasite growth in this model system could be

useful for understanding the differences in multiplication rate of isolates of *P. falciparum*, which as shown earlier, can also be associated with disease.

There are two approaches which can be used to investigate which parasite genes are involved in determining phenotypes, such as pathogenicity. The first approach is to speculate as to the most likely candidates and then search for associations between these candidates and the infection phenotype in a large number of parasites. However, as described above, this approach has been taken in field studies of *P. falciparum* disease severity and has not been very successful, presumably because there are so many genes and other factors which could influence this phenotype. The second approach is to take a population of different parasites and type them for their phenotype and the inheritance of many genetic markers, using genetic linkage to find candidate genes for the phenotype of interest. As this latter approach does not require initial speculation, genetic linkage analysis is a more elegant and efficient way of determining the genetic basis of phenotypes such as growth, where there is little prior evidence of the involvement of specific candidate genes. Genetic linkage analysis is the method which will be used to investigate the genetic basis for the difference in parasite growth properties in this thesis.

1.6. *Plasmodium* Genetics

1.6.1. Genetic Crosses

The ability to carry out genetic linkage analysis depends on the organism of interest undergoing sexual reproduction, or meiosis, in which recombination between two

genomes occurs. The lifecycle of the haploid malaria parasite is such that it undergoes meiosis only in the mosquito vector with the fusion of male and female gametes to form a diploid zygote. If gametes from the same malaria parasite strain are taken up, "selfing" occurs, producing meiotic progeny which are homozygous. If the host is infected with malaria parasites of different strains, gametes from these different strains can cross-fertilise, producing heterozygous progeny. Random mating was found to occur in *P. falciparum*, indicating that there was no preference for either crossing or selfing (Ranford-Cartwright et al. 1993).

In the 1950s, several attempts were made to establish whether crosses could be made between strains of the avian malaria parasite *P. gallinaceum* (Greenberg and Trembley 1954a, 1954b; Trembley and Greenberg 1954; Greenberg 1956). However, due to the lack of genetic markers and distinct phenotypes available at the time, genetic crossing between these parasites was not proven conclusively. It was not until 1971 that the first genetic cross between two malaria parasites was fully demonstrated in the laboratory, using two strains of the rodent malaria parasite *P. yoelii* (Walliker et al. 1971). This was followed in 1975 with the successful crossing of two strains of another rodent malaria parasite, *P. chabaudi* (Walliker et al. 1975).

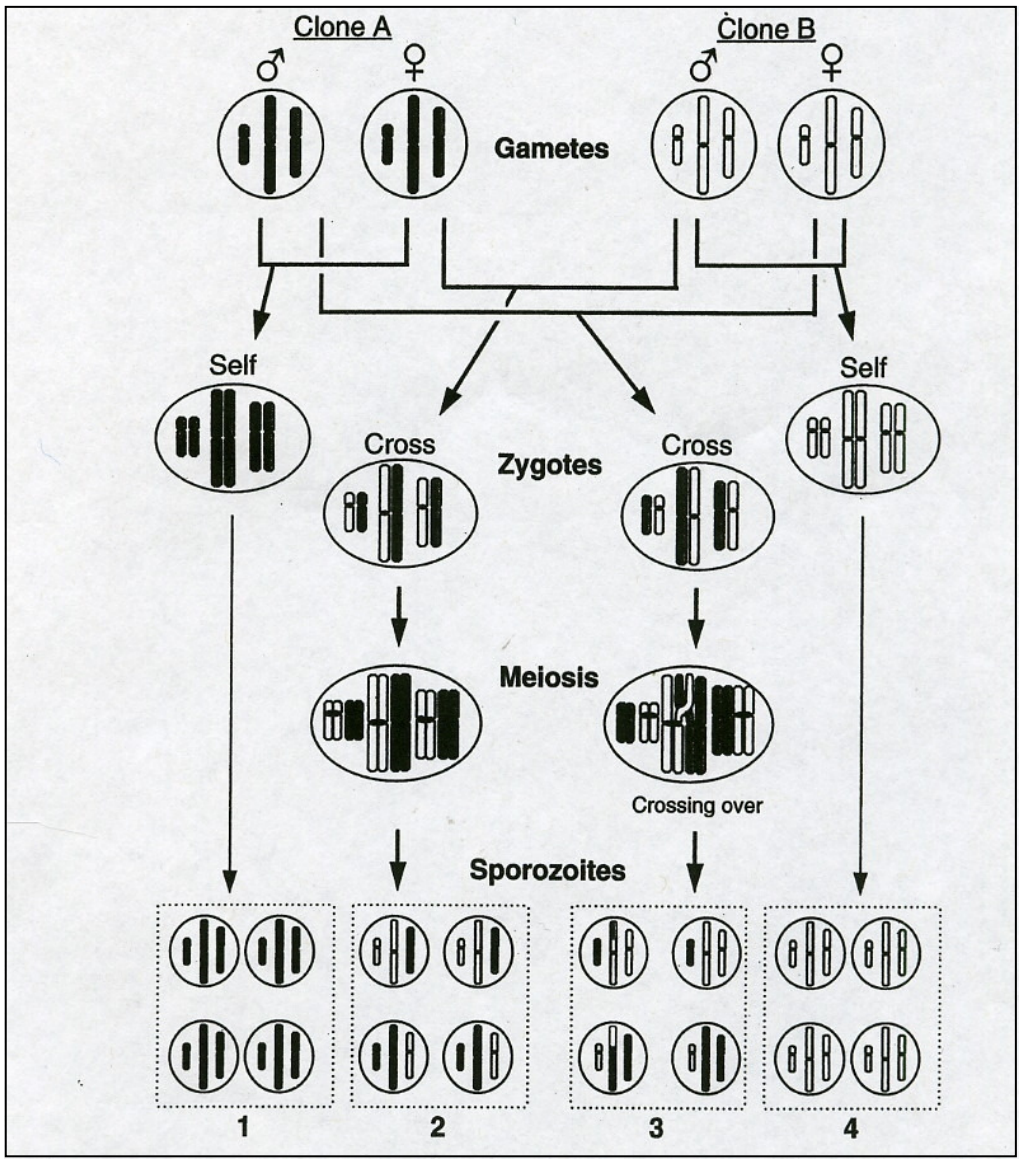
However, investigations of genetic recombination in *P. falciparum* had to wait several more years until the advent of *in vitro* culture methods and membrane-feeding techniques allowed the maintenance of the parasite lifecycle in the laboratory. However, as there was no *in vitro* system for the sporozoite infection of hepatocytes to generate merozoites, splenectomised chimpanzees were used.

Unfortunately this is still the situation today, and as the use of primates has substantial financial and ethical considerations, only three crosses have been carried out to-date between different strains of *P. falciparum*. The first cross, published in 1987, demonstrated recombination between *P. falciparum* strains 3D7 and HB3 (Walliker et al. 1987), the second was a cross between HB3 and another strain Dd2, published in 1990 (Wellems et al. 1990). The third cross, between *P. falciparum* 7G8 and GB4, has been carried out more recently but is as yet unpublished (described in (Su et al. 2007).

Recombination at meiosis can take the form of either (1) homologous chromosome reassortment, or (2) crossing-over events between homologous chromosomes (Figure 1.5). In addition to these processes, in *P. falciparum*, recombination has been shown to occur between the sub-telomeric regions of heterologous chromosomes, leading to ectopic chromosome rearrangement (Freitas-Junior et al. 2000). Similarly, copy number polymorphisms of many genes have been found by comparing several *P. falciparum* laboratory and field isolates (Ribacke et al. 2007), indicating that this might be a further mechanism of generating genetic diversity during replication.

In an early study in *P. falciparum*, the average recombination distance in *P. falciparum* was found to be approximately 15-30kb per cM (where 1 cM = 1% frequency of recombination between 2 markers) (Walker-Jonah et al. 1992). This is in agreement with a later study using the same genetic cross progeny and larger numbers of markers, which found there was an average 17kb per cM (Su et al. 1999). In this analysis, gene deletion, translocation, and non-reciprocal gene conversions

Figure 1.5
Genetic recombination, by chromosome reassortment and crossing-over at meiosis, between the gametes of two haploid malaria parasites “clone A” and “clone B” (image reproduced from (Walliker et al. 1998).



were also identified. In addition, the rate of recombination by crossing over was found to be uniform with respect to physical distance across the genome in this study (Su et al. 1999). However, in a later study of population genetic data from a large number of worldwide *P. falciparum* isolates, using a high density of genetic markers on one chromosome (chromosome 3), recombination rates were found to vary across the chromosome, with "hot-spots" in the middle and in the subtelomeric region at each end of the chromosome (Mu et al. 2005). This is not surprising given that variation in recombination rates across the genomes of other eukaryotic organisms is very common (Nachman 2002), however, a further investigation of this phenomenon across the whole of the *P. falciparum* genome would be beneficial.

1.6.2. Genetic Linkage Analysis

Recombination occurs between genetically distinct *P. falciparum* parasites in natural populations in the field, leading to high levels of genetic complexity and population structure (reviewed in (Walliker et al. 1998). Geographical variation in the frequency of multiple infections and the rate of transmission means that the effective recombination rate differs between parasite populations; African *P. falciparum* populations were found to have very high cross-fertilisation rates, whereas South American populations had comparatively lower rates (Conway et al. 1999; Mu et al. 2005). This means that underlying differences in transmission and recombination rates between populations could confound association studies of particular parasite gene alleles to phenotypes, such as those described in section 1.3 of this introduction.

Despite these differences it is possible to identify recent selection on specific parasite genes in the population, such as those determining drug resistance. This is because a "selective sweep" occurs as a beneficial mutation is driven rapidly through the parasite population in the face of strong drug selection pressure. Linkage disequilibrium, between genetic markers flanking drug resistance genes, occurs as these genetically linked, but functionally neutral loci, "hitch-hike" along with the drug resistance gene (e.g.(Wootton et al. 2002; Nair et al. 2003; Mu et al. 2005) and reviewed in (Walliker 2005). Due to the close physical linkage of these markers to drug resistance genes, recombination will only rarely break down this linkage disequilibrium, the exact frequency depending on the effective recombination rate in the parasite population. Therefore, the detection of a region of marker linkage disequilibrium in a parasite population can identify the target of a recent selection pressure. For example, reduced microsatellite marker diversity in a Thai parasite population was found around the *dhfr* gene, which was presumably responsible for the pyrimethamine-driven selective sweep (Nair et al. 2003).

In the laboratory, recombination between malaria parasites of the same species but differing phenotypes can be exploited to identify the genetic basis of those phenotypes. Classical genetics studies using cloned progeny from the two *P. falciparum* crosses published to date, have found genes or linkage groups which were associated with, for example, chloroquine resistance (Wellems et al. 1991; Su et al. 1997), pyrimethamine resistance (Peterson et al. 1988), quinine resistance (Ferdig et al. 2004) and invasion/growth properties (Wellems et al. 1987; Peterson and Wellems 2000). Unfortunately, as crosses in *P. falciparum* currently require the use

of primates, the potential for classical genetic analysis in the human malaria parasite remains largely untapped. Using malaria parasite infections of rodents to generate crosses for genetic analysis is a more experimentally tractable method, and several crosses have been made and analysed using a classical genetics approach. These have identified genetic loci involved in, for example, chloroquine resistance (Carlton et al. 1998a; Hunt et al. 2004), mefloquine resistance (Cravo et al. 2003) and sulphadoxine-pyrimethamine resistance (Hayton et al. 2002) in *P. chabaudi*, and in the difference in growth properties between two strains of *P. yoelii* (Walliker et al. 1976).

1.6.3. Genomics

Recent advancements in *Plasmodium* genomics have increased our ability to undertake and interpret genetic analyses. The sequence of the *P. falciparum* genome was first published in 2002, comprising around 23Mb of sequence on 14 nuclear chromosomes (Gardner et al. 2002). It is extremely A-T rich; around 80% of the genome is made up of A or T nucleotides, and around two-thirds of the 5300 proteins encoded by the genome are unique to *Plasmodium*. Malaria parasites also have two extra-nuclear genomes; the 35kb apicoplast genome and the 6kb mitochondrial genome. The genomes of rodent malaria parasites used in genetic analyses have also received attention. The 17Mb *P. chabaudi* nuclear genome of the AS strain was published to 3x coverage in 2005 (Hall et al. 2005) but coverage has since been increased to 8x (http://www.sanger.ac.uk /Projects/P_chabaudi/). The genome sequences of *P. berghei* and *P. yoelii* have also been published and are now at 8x and 5x coverage respectively (http://www.sanger.ac.uk/Projects/P_berghei/ and

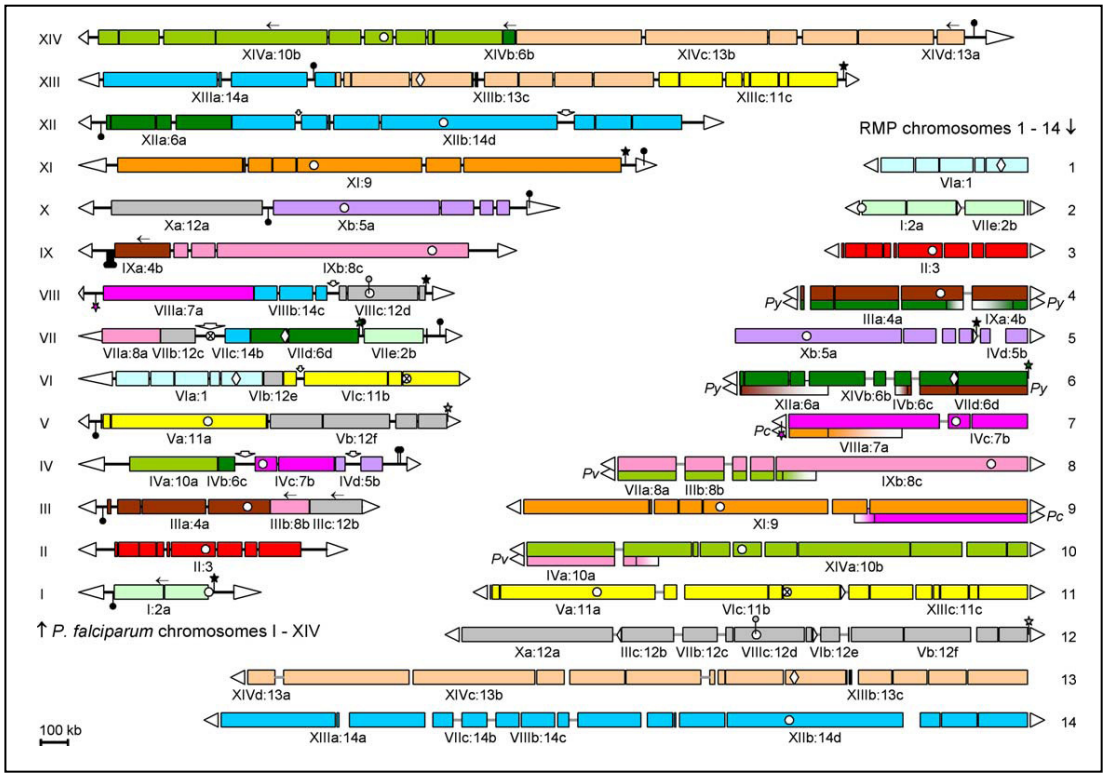
(Carlton et al. 2002). However, none of the rodent malaria parasite genomes has yet been fully assembled and annotated. In practice, this means that the databases are comprised of many contigs (sections of genome sequence) which are not joined to each other because gap-closure is still on-going.

A large proportion, around 80%, of rodent malaria parasite genes have orthologous genes in *P. falciparum* and these are distributed across the core (non-subtelomeric) regions of all 14 *P. falciparum* chromosomes (Hall et al. 2005; Kooij et al. 2005). Gene order is conserved in these blocks of orthologous sequence, although the chromosomal location of these regions varies, indicating genetic synteny between the species of *Plasmodium*. This confirms previous reports of gene synteny observed in *Plasmodium* (Carlton et al. 1998b; Hunt et al. 2004). This genetic synteny can be exploited; using the fully assembled *P. falciparum* genome sequence as a scaffold, contig sequences from the rodent malaria parasites *P. chabaudi*, *P. yoelii*, and *P. berghei* have been mapped to orthologous locations in *P. falciparum*, generating a synteny map (Kooij et al. 2005) and Figure 1.6. This has created a workable "pseudo-assembly" for genetic analysis in rodent malaria parasites.

1.6.4. Linkage Group Selection (LGS)

Classical genetic linkage analysis in *P. falciparum* and rodent malaria parasites, as described above in section 1.6.2, requires the production of many cross progeny clones which are screened for the inheritance of parental phenotypes of interest, such as drug resistance. These clones are then typed for the inheritance of genetic markers

Figure 1.6
Synteny map between *Plasmodium falciparum* and the rodent malaria parasites *Plasmodium chabaudi*, *Plasmodium yoelii* and *Plasmodium berghei*, as a composite genome (RMP) (image reproduced from (Kooij et al. 2005).



on different chromosomes, and the linkage of particular markers to a particular phenotype is calculated. The advantage of this approach is that it is discovery orientated and not hypothesis driven. This is unlike other genetic studies where a candidate gene for a particular phenotype is chosen, and then tested for association with the phenotype in a particular parasite population. However, generating and phenotyping large numbers of cross progeny clones is very time consuming. Large numbers of genome-wide markers must also be screened in individual cross progeny clones, in order for any linkage to be discovered. The shortcomings of classical genetic linkage analysis have led this laboratory to develop Linkage Group Selection (LGS), a novel genetic technique for rapid gene discovery in rodent malaria parasite infections (Carter et al. 2007).

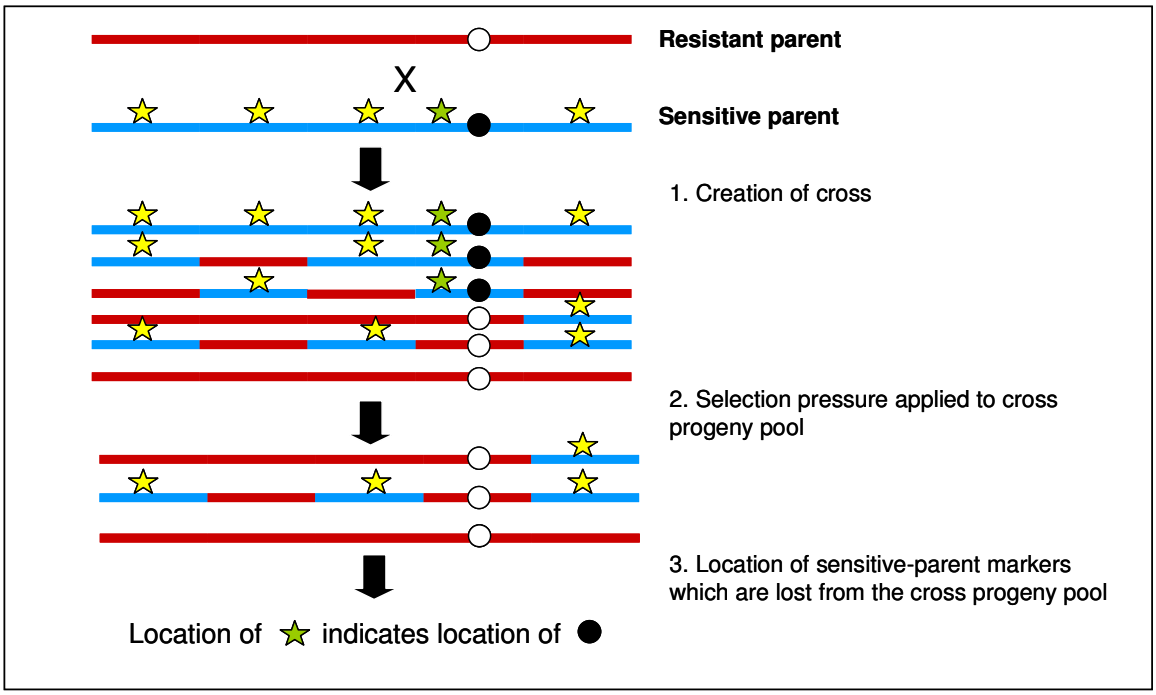
LGS has two advantages. Firstly, as a method of genetic analysis using rodent malaria parasites, it benefits from the availability of substantial genomic information, the relative ease of conducting genetic linkage analysis in the rodent experimental system and the large number of distinct rodent malaria parasite strains which are available. Moreover, the large number of orthologous genes shared by the rodent malaria parasites and *P. falciparum* means that discoveries from this approach might be translated to the human malaria parasite. Secondly, as an approach which is different to the classical genetic linkage analysis method described above, it is much faster and economical; there is no need to clone out and individually characterise recombinant cross progeny.

As described earlier, the rodent malaria parasite strains differ in several interesting phenotypes which can now be fully explored with a time- and cost-effective genetic linkage approach like LGS. LGS involves three main steps (Carter et al. 2007) and is illustrated in Figure 1.7. The first step is to make a cross between two rodent malaria parasite strains that differ in a selectable phenotype of interest, e.g. drug resistance. The two strains, "sensitive" and "resistant", are simultaneously inoculated into a mouse, and the subsequent mixed infection transmitted to *Anopheles stephensi* mosquitoes from a laboratory colony. At meiosis, genetic recombination occurs between gametocytes of the same strain, and between gametocytes of different strains. This generates parental-type and recombinant cross progeny in equal numbers, providing conditions are optimal.

The second step is to dissect a large number of infected mosquitoes, removing the salivary glands containing the cross progeny sporozoites, and pooling them all together. This pool of sporozoites is then directly injected into a mouse to establish a blood infection. This pool of uncloned cross progeny is then subjected to a specific selection pressure, for example, exposure to an anti-malarial drug. The parasites surviving the selection pressure are termed the "uncloned, selected cross progeny".

The third step is to take these uncloned, selected cross progeny and to screen the pool for the inheritance of quantitative markers which are specific for either parent and located across the genome. The genetic markers which have been used for LGS analysis thus far are either amplified fragment length polymorphism (AFLP) markers (Vos et al. 1995; Grech et al. 2002; Martinelli et al. 2004) or single nucleotide

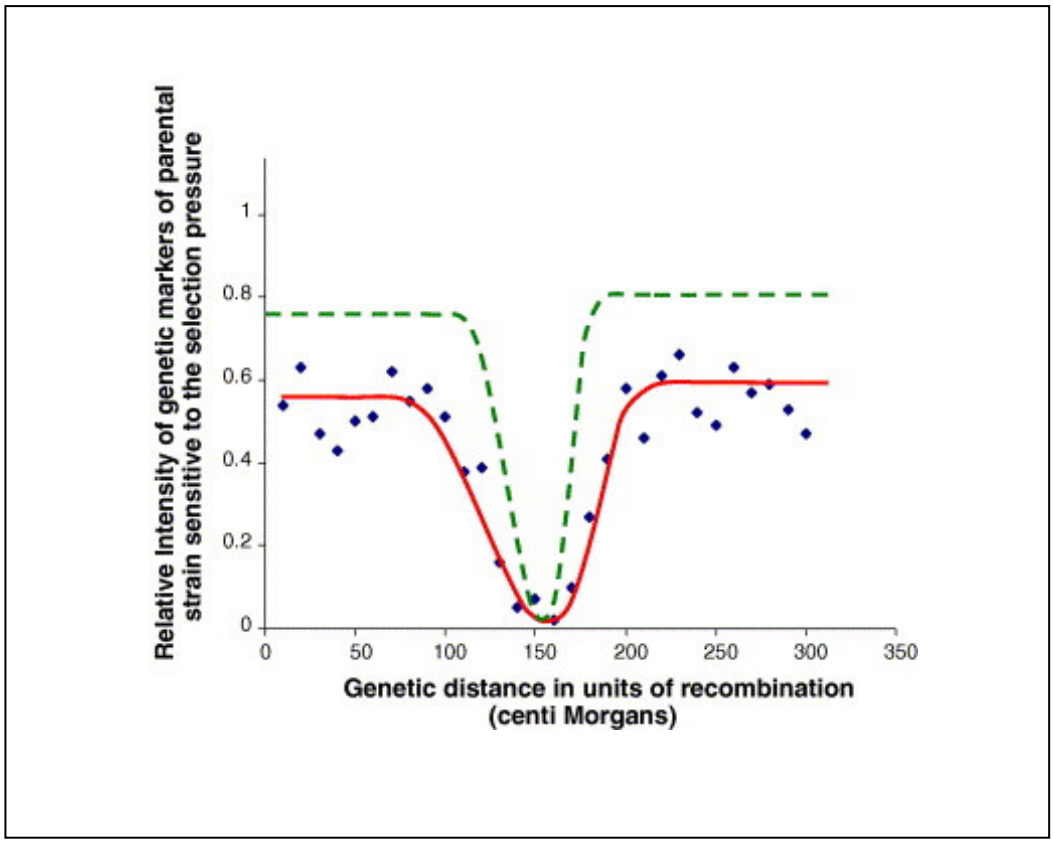
Figure 1.7
The principle of Linkage Group Selection (LGS).
The genome of the "resistant" parent is shown as a single red line; the genome of the "sensitive" parent is shown as a single blue line. A white circle represents the location of a gene conferring the "resistant" phenotype; a black circle represents the location of a gene conferring the "sensitive" phenotype. The locations of genetic markers specific to the "sensitive" parent are shown as yellow stars; the green star represents the most closely-linked genetic marker to the gene controlling the phenotype. LGS involves 3 main steps: 1) The two strains are crossed in the mosquito vector and undergo recombination at meiosis, generating cross progeny which have either recombinant or parental-type genomes; 2) A selection pressure is applied to the cross progeny pool and only those which have inherited the gene conferring resistance to the selection pressure (white circle) will be present after selection; 3) The location of the gene conferring the "sensitive" phenotype (black circle) can be found by locating genetic markers specific to the "sensitive" parent which are reduced in representation in the cross progeny pool following selection (green star).



polymorphism (SNP) markers measured by Pyrosequencing™ (Ronaghi et al. 1998; Cheesman et al. 2007). These techniques are described in more detail below, as both have been employed in this thesis. After the application of the selection pressure, any cross progeny which have inherited the gene which is the target of the selection pressure, will be lost from the cross progeny pool. These would be any parasites which are of the parental-type which is "sensitive" to the selection pressure, and any recombinant parasites which have inherited the particular genetic locus from the "sensitive" parent. Markers specific to the "sensitive" parent and which are linked by close physical distance to the gene under selection, will also be lost from the cross progeny pool. Markers specific to the "sensitive" parent and which are sufficiently far away from the target locus to be unlinked, will not be lost following selection.

The chance of a recombination event occurring between the marker and the target locus increases with distance, leading to a gradual loss of genetic linkage and therefore, a gradual increase in marker inheritance. Measuring the inheritance of "sensitive" parent markers therefore produces a "selection valley" around the gene which is the target of the selection pressure (Figure 1.8). Therefore, the LGS approach uses the same principle employed by population genetics studies in *P. falciparum*, in which regions of linkage disequilibrium between genetic markers can reveal the targets of selective sweeps, as described above in section 1.6.2. A similar technique, using AFLP markers to type uncloned populations of selected cross progeny, has been used for the identification of genetic loci responsible for strain-specific protective immunity in the related Apicomplexan parasite *Eimeria maxima* (Blake et al. 2004; Blake et al. 2006).

Figure 1.8
A theoretical "selection valley" around a gene which is the target of a selection pressure in an LGS analysis.
The proportion of a cross progeny pool (red line) and a backcross progeny pool (green line) which are carrying genetic markers specific to the sensitive parent, after a selection pressure has been applied (image adapted from (Carter et al. 2007).

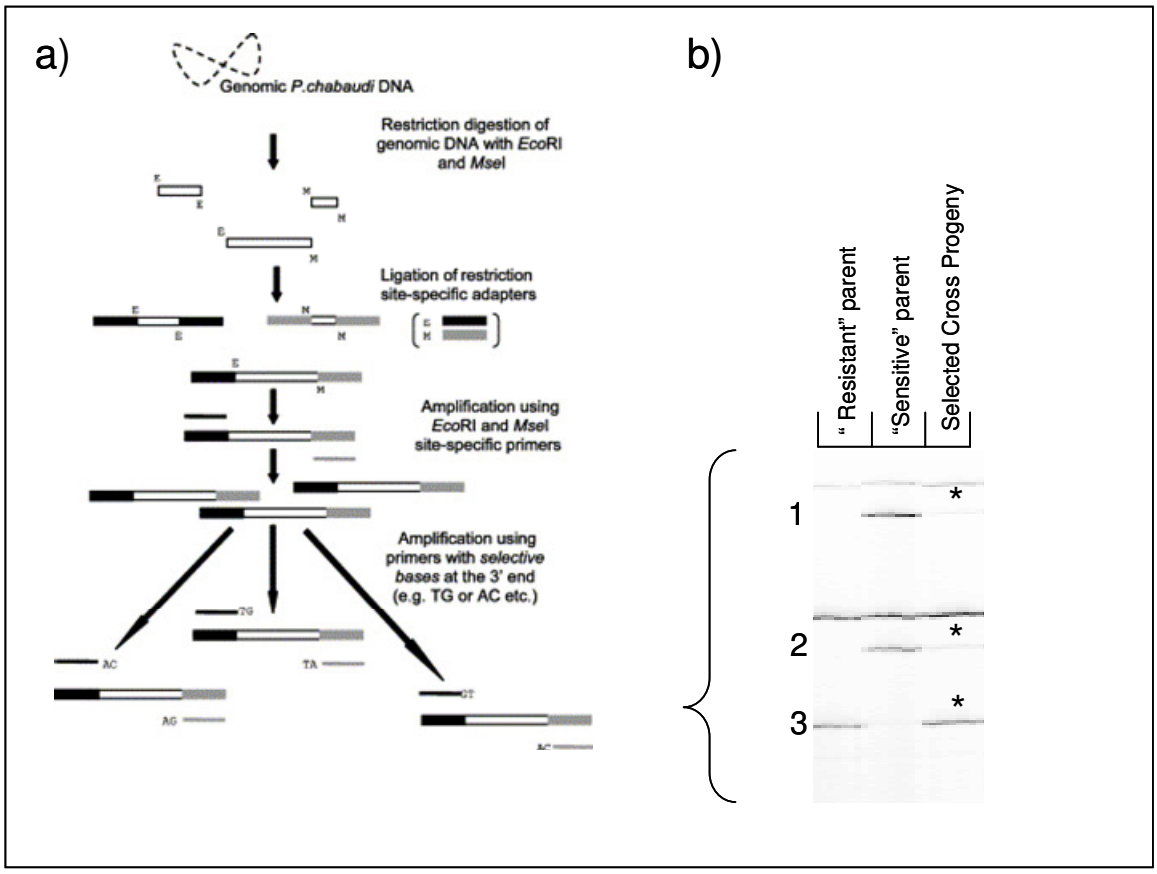


1.6.5. Measuring Marker Inheritance for LGS Analysis

The key to the LGS method is the availability of quantifiable, genome-wide markers. The first method employed in this thesis is proportional AFLP, which has been described and validated by this laboratory for the quantification of the proportion of parasites in a mixture carrying a particular AFLP marker (Grech et al. 2002; Martinelli et al. 2004). Genomic DNA from the pure parental strains and the pooled progeny resulting from a cross between these two strains, is digested with a frequent-cutting restriction enzyme like MseI and a rare-cutting restriction enzyme like EcoRI (Figure 1.9a). Adaptors specific to these cleavage sites are then attached to the ends of the genomic DNA fragments. Subsets of DNA fragments from this pool are amplified by PCR using primers which bind to the adaptors and which have 2 selective nucleotides at the 3' end, e.g. AA, AC, AG, etc. This step is repeated several times with primers that have different selective nucleotides, to amplify several different pools of DNA fragments. DNA pools are radio-labelled with a radioisotope of phosphorous, ^{33}P, and run on polyacylamide gels by electrophoresis, to separate the fragments on the basis of size (Figure 1.9b).

This technique generates specific "finger-prints" of the two parent parasite strains used in the cross. Where a polymorphism in an enzyme restriction site has occurred between the two strains and a band of particular size is present on the gel for one strain, but not the other, this denotes a marker specific for one of the parents. The relative presence or absence of these markers (the Relative Intensity) in the cross progeny pool after selection, compared to the parental strain, can be determined quantitatively. The dried gel is exposed to a phosphoscreen and then the image is

Figure 1.9
The AFLP method
a) Genomic parasite DNA from each parental strain and from cross progeny pools is digested with EcoRI and MseI restriction enzymes and fragments of DNA are selectively amplified by PCR (image reproduced from Grech et al, 2002). b) The ^{33}P – labelled products of each selective PCR reaction are separated by polyacylamide gel electrophoresis and bands specific to each parental strain (markers) can be visualised (1 - 3). The inheritance of these parent-specific AFLP markers can be quantified in the cross progeny pool (*).



scanned in and the intensity of individual marker bands can be measured using computer software. The advantage of this technique is that it requires no prior knowledge of the genome sequence, because markers are simply made by restriction enzyme digestion. Once markers of interest have been identified, the DNA fragments can be cut out from the gel, sequenced and located on genetic linkage maps, or in the genome, if available (Martinelli et al. 2005a). However, a disadvantage of this technique is that if the marker inheritance is around 10% or lower, the quantification becomes less accurate. It is also a fairly laborious process and requires the use of radioisotopes.

Therefore a second method, Pyrosequencing™ (hereafter referred to as pyrosequencing), has also been employed in this thesis. This technique has recently been validated in this laboratory and accurately quantifies the proportion of parasites in a mixture carrying a particular single nucleotide polymorphism (SNP) marker (Cheesman et al. 2007). Unlike proportional AFLP, in which the marker locations are not known initially, these SNP markers are designed at specific points within the genome sequence of *P. chabaudi*, using the *P. falciparum* synteny map (Kooij et al. 2005). Once a particular location is chosen, primers are designed using Primer3 web-based software (http://primer3.sourceforge.net/) to amplify a fragment of *P. chabaudi* sequence around 500bp in size from each of the parental strains. These sequences are then aligned using ClustalW2 web-based software (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and any SNPs between the two strains are identified. Pyrosequencing Assay Design Software (Biotage) is used to

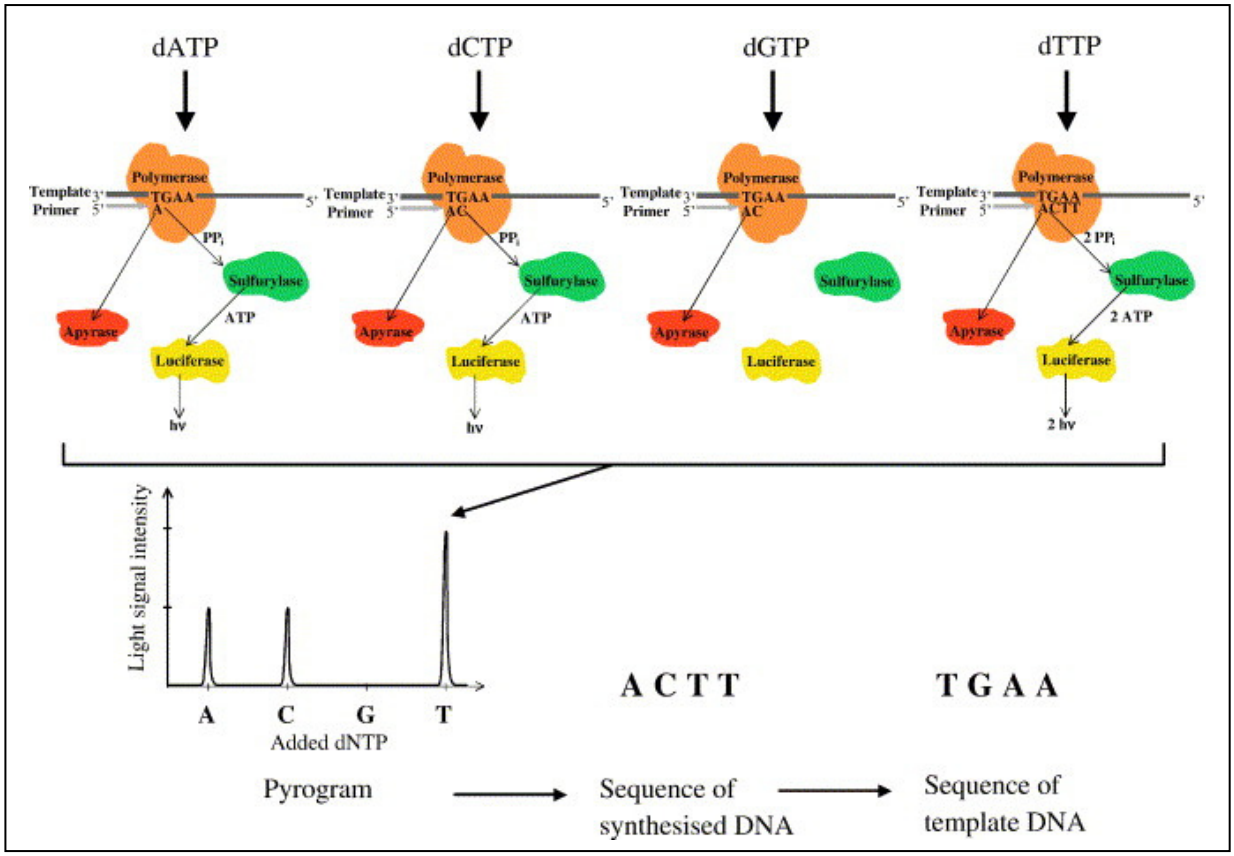
determine which SNP would give the most accurate allele quantification and to design primers around this marker SNP for use in the pyrosequencing assay.

From the cross progeny pool, a small fragment of DNA around the marker SNP is amplified. The DNA is then made single-stranded and a short sequencing primer attached to the DNA template in front of the marker SNP. Nucleotides are then added sequentially in a "sequencing by synthesis" reaction in which the successful addition of a complementary nucleotide to the template results in the release of pyrophosphate (PPi) (Figure 1.10). This reacts with a substrate, producing a light signal in proportion to the amount of nucleotide incorporated. At the marker SNP, the proportion of the cross progeny DNA pool which incorporates either parent-specific nucleotide can be determined by the amount of light emitted when each of the specific nucleotides are added. Pyrosequencing is a rapid way of measuring marker inheritance in an LGS analysis because once all the markers are designed, the system is high through-put. It is also more accurate than AFLP at determining low levels (<10%) of marker inheritance.

1.6.6. LGS Analysis of Differences in Growth

This thesis details an investigation into the genetic basis of the difference in the growth properties of two rodent malaria parasite strains, using the LGS approach described above. Previous, published LGS analyses have investigated the genetic basis of drug resistance and strain specific protective immunity (Culleton et al. 2005; Martinelli et al. 2005b; Hunt et al. 2007; Pattaradilokrat et al. 2007). However, the interesting differences in growth and pathogenicity between rodent malaria parasite

Figure 1.10
The principle of the pyrosequencing reaction
A primer is annealed to a single-stranded DNA template, in the region of a SNP differentiating two strains. A polymerase adds nucleotides one at a time; the successful incorporation of a nucleotide leads to the release of pyrophosphate (PPi) which reacts with sulfurylase to produce ATP. The ATP reacts with luciferase to produce a photon; the light emission is recorded on a pyrogram as a peak with a height relative to the amount of nucleotide incorporated. Apyrase removes unincorporated nucleotides. At the SNP, the proportion of the DNA templates incorporating either nucleotide is quantified by the software program from the relative peak heights. (Image reproduced from (Ahmadian et al. 2006).



strains have only recently begun to be investigated. An LGS analysis of the difference in the growth properties of two strains of *P. yoelii*, 33X and YM, has found that a single genomic region is important (Pattaradilokrat 2008), confirming the interpretation of an earlier classical genetic linkage study (Walliker et al. 1976).

The lethal, rapid growth property of YM arose during maintenance in the laboratory of a slow-growing, non-lethal strain. In contrast, there are quite large differences in the growth properties of several *P. chabaudi* strains, which were present at the time of isolation from the wild and which have been stable over time. Moreover, the *P. yoelii* YM strain does not have the strong preference for reticulocyte invasion which is typical of non-lethal *P. yoelii* strains, whereas there are no visible differences in erythrocyte invasion that would explain the different growth properties of strains of *P. chabaudi*. Therefore, it was interesting to investigate growth differences between *P. chabaudi* strains particularly, using the LGS approach.

Two strains of *Plasmodium chabaudi adami*, DK and DS, were derived from rodent malaria parasite isolates 556KA and 408XZ respectively, extracted from thicket rats (*Thamnomys rutilans*) captured in the Congo (Brazzaville) in 1970 and 1972 (Carter and Walliker 1976; Carter 1978). A recent phylogenetic analysis of the four rodent malaria parasite species using DNA sequence data from 7 genes confirms the species/subspecies groupings based on morphological observations and isozyme analysis (Perkins et al. 2007). The two *P. c. adami* isolates 408XZ and 556KA group separately to strains of the other subspecies, *P. c. chabaudi*. However, as expected,

they are more closely related to strains of *P. c. chabaudi* than to those of *P. berghei*, *P. yoelii* or *P. vinckei*.

DS and DK were chosen for LGS analysis, from the range of other *P. chabaudi* strains available, on the basis of the very large difference in growth between them, and the association of this with pathogenicity; in the experience of this laboratory, DS is a fast-growing, pathogenic strain and DK is a slow-growing, non-pathogenic strain. These characteristics were apparent in the original thicket rat isolates (Carter and Walliker 1976) and are evident in other laboratories in various infection settings (Snounou et al. 1992; Crewther et al. 1996; Anders et al. 1998; Xu et al. 2000; Hernandez-Valladares et al. 2004; Scorza et al. 2005). There can be little doubt, therefore, that the differences in growth between these two parasite strains are stable and innate characteristics of the parasites themselves.

For an LGS analysis of growth differences, the method is slightly modified from that described in section 1.6.4. The selection pressure that is applied to the cross progeny is simply to let fast-growing recombinants outgrow slower ones over several rounds of blood infection. Slow-growing recombinants and the slow-growing parent, and thus markers associated with slow growth, are removed from the cross progeny pool in this way. Identification of these markers holds the key to discovering parts of the genome conferring the growth phenotype.

In describing the differences in growth properties between DS and DK in this thesis, it has been necessary to avoid the use of the phrases “growth rate” or “multiplication

rate". As will be demonstrated in Chapter 2, DS and DK reach maximum parasitaemia around the same day of infection, but DS reaches a much higher parasitaemia than DK. The expansion in parasitaemia per day, the true "growth rate", is not constant over the course of infection with either strain, and this reflects the nature of parasite growth *in vivo*. Therefore, although DS is described in this thesis as fast-growing and DK as slow-growing, this is based on the magnitude of the parasitaemia reached over a number of days of infection, rather than on the strict definition of "growth rate". This property is distinct from that investigated in studies of *P. falciparum* which describe the *in vitro* "multiplication rate" of particular isolates, i.e. the number of newly infected red blood cells observed after a single erythrocytic cycle in *in vitro* culture (Chotivanich et al. 2000; Deans et al. 2006).

1.7. Aims of the Project

The first aim was to fully describe the parasitological differences between *P. c. adami* DS and DK which are relevant to the LGS investigation. These were the differences in parasitaemia over the course of infection, in pathogenesis, in selectivity of red blood cell invasion, in gametocyte production and in infectivity to mosquitoes, between single infections of the two strains. In addition, it was important to describe the growth properties of the two strains whilst together in mixed infections because the creation of mixed infections is a necessary part of the LGS technique. These experiments are detailed in Chapters 2 and 3.

The second aim was to carry out the LGS analysis of the genetic basis for the difference in the growth properties of DS and DK. This involved the successful

crossing of the two strains and the selection of cross progeny for the fast-growth phenotype of DS. This was carried out on several occasions, generating several independent crosses and backcrosses, in order to increase the confidence of the genetic analysis. Growth-selected cross progeny pools were then screened for the inheritance of genome-wide AFLP markers specific to the DS or DK strain. The inheritance of SNP markers specific to the DS or DK strain was also measured, using pyrosequencing, in genomic regions identified by the genome-wide AFLP marker screen. This fine-mapping allowed regions of the genome under selection to be more closely defined. These experiments are detailed in Chapters 4 and 5. Further investigations were undertaken to confirm that the selection against these genetic regions was occurring following growth of cross progeny in the blood and not at any other stage of the lifecycle. These experiments are detailed in Chapter 6.

The third aim was to identify the potential candidate gene(s) in the genetic loci which are linked to the determination of the different growth properties of *P. c. adami* strains DS and DK. Due to the nature of the genomic regions identified by LGS analysis, it was not possible to narrow these regions down to candidate genes within the time-scale of this project. This matter is discussed in detail in Chapters 5 and 6.

Chapter 2: Growth, Pathogenicity and Transmissibility of *P. c. adami* DS and DK

2.1. Foreword

Before embarking on the main objective of this thesis; the genetic analysis of the growth difference between *P. c. adami* DS and DK, it was necessary to fully describe these growth differences. Therefore, the differences in growth, pathogenicity and selectivity of RBC invasion between DS and DK are examined in this second chapter. This information will help to identify the correct timing for the selection of DS x DK cross progeny pools for fast growth. In the course of a genetic analysis by LGS, it is necessary to transmit the two parasite lines of interest to mosquitoes, a process that, in the experience of this laboratory, can depend significantly on the timing of infection and the parasite and host genotypes used. Therefore, this chapter also includes a study on the numbers of gametocytes produced and the transmission success of both DS and DK, over time in two different mouse host strains. From these results it will then be possible to optimise the conditions for creating crosses between DS and DK, to generate large numbers of recombinants for genetic analysis.

In the process of this investigation it was evident that the findings relating to the pathogenicity and transmission success of these strains contrasted with the literature on the related subspecies, *P. c. chabaudi*. Therefore, a version of this chapter was submitted in March 2008 to the International Journal for Parasitology and was accepted for publication in July 2008. It is currently in press.

2.2. Introduction

Several genetically distinct strains of the rodent malaria parasite *Plasmodium chabaudi* were isolated from wild-caught thicket rats captured in central Africa in the 1960s and 1970s (Carter and Walliker 1975, 1976). In laboratory mice, strains of the *P. c. chabaudi* subspecies that reach higher maximum parasitaemias tend to be more pathogenic (causing more weight loss and anaemia), than those that achieve lower parasitaemias (Mackinnon and Read 1999; Mackinnon and Read 2004).

Further studies have found that the pathogenicity and transmissibility to mosquitoes of *P. c. chabaudi* strains are highly correlated with each other (Mackinnon and Read 1999; Ferguson et al. 2003). Since these differences in growth and pathogenicity were found when infections were studied in the same strain of inbred mice, genetic differences between the parasite strains themselves, rather than host variation, must be major determinants of the differences in infection phenotype.

The following study is an investigation of the relationship between growth, pathogenicity and transmissibility to mosquitoes in a sister subspecies of these rodent malaria parasites, *P. c. adami*, which will be used in the genetic analysis described later in this thesis.

2.3. Materials and Methods

2.3.1. Parasites, Mice and Mosquitoes

Non-pathogenic and pathogenic strains of the rodent malaria parasite *P. c. adami* were derived from two parasite isolates, 556KA and 408XZ, respectively. These isolates were made by sub-inoculation of blood from wild-caught thicket rats (*Thamnomys rutilans*) of the Congo (Brazzaville) into laboratory rodents (Carter and Walliker 1976). The parasites were cloned, and the cloned lines of DK (slow-growing) and DS (fast-growing) are the material used in the present work. These cloned lines are referred to hereafter as strains DK and DS respectively.

6-8 week old female CBA/Ca or C57BL/6J mice were used for blood-stage infections. These mouse strains will be referred to hereafter as CBA and C57 respectively. To generate a blood stage infection, a small volume of donor mouse blood containing 10^6 parasitised red blood cells (RBCs) was mixed in 0.1ml 50% Fetal Calf Serum 50% Ringers solution and injected intra-peritoneally into a recipient mouse.

Female *Anopheles stephensi* mosquitoes used for transmission of the parasites were from a laboratory colony. The adult mosquitoes were maintained at 26-27°C and 60-90% relative humidity and fed with 10% glucose in water with 0.05% *para*-amino benzoic acid. All animal experiments in this thesis were carried out in accordance with the Animals (Scientific Procedures) Act 1986, UK.

2.3.2. Characterisation of Blood Infections

To characterise the growth of the parasites in the blood of infected mice, blood smears from each of two groups of 5 female CBA mice infected with 10^6 DS or DK

parasitised RBCs were examined daily for parasitaemia, gametocytaemia and selectivity of red blood cell invasion. The mean and standard error of the mean (sem) of all these measurements were calculated for the five mice in each group. Data were normally distributed and were compared using the Student's *t*-test.

Blood smears (1 per mouse per day), were made with a thin smear of tail blood on a microscope slide, fixed with methanol and stained with a solution of 10% Giemsa's stain in PBS pH 7.2 for 30 minutes. *P. chabaudi* infections are synchronous, and blood smears were taken between approximately 9am and 11am each day, when all parasites were at the ring stage of development (early trophozoites). Five fields of vision (at 1250x magnification with oil immersion) in which the RBCs were accurately counted, representing an average of 1000 RBCs per slide, were examined for the percentage RBCs infected to give the parasitaemia. To determine gametocytaemia, 100 fields of vision representing approximately 20,000 RBCs (not individually counted) were examined per slide to give the percentage RBCs containing gametocytes. The number of RBCs/ml was estimated by taking a 2µl tail blood sample, mixing with 80ml Isoton (Beckman Coulter Inc) and using a Coulter cell counter (Beckman Coulter Inc). Gametocyte conversion rates were measured by dividing the gametocytaemia on a given day (day n) by the asexual stage parasitaemia on the previous day (day n-1)**,** because *P. chabaudi* gametocytes take 24 hours to mature.

Selectivity Indices were determined according to the method of Simpson and others (Simpson et al. 1999); 300 parasitised RBCs were counted per slide and the number

of multiply-infected cells (>1 parasite per cell) was recorded. The mean number of parasites per RBC (the parasite density per RBC) was also determined by counting the number of parasites in approximately 1000 RBCs. The number of multiply-infected RBCs expected by chance, given the parasite count, was determined from the Poisson distribution by the following equation:

$$\mathbf{P_x = e^{-\mu} \mu^x / x!}$$

Where **x** is a particular number of parasites per RBC, **μ** is the parasite density per RBC, and $\mathbf{P_x}$ is the probability of **x** parasites per RBC.

For example, if there are 172 parasites in 1027 RBCs, **μ** = 172/1027 = 0.167

Therefore, $\mathbf{P_x}$, where **x** = 0, and **μ** = 0.167, is 0.846

i.e. given the parasite density per RBC, the probability of 0 parasites per RBC is 0.846

Therefore, the probability of any number of parasites per RBC is: 1 - 0.846 = 0.154

$\mathbf{P_x}$, where **x** = 1, and **μ** = 0.167, is 0.141

i.e. the probability of 1 parasite per RBC is 0.141

Therefore, the expected number of the 300 pRBCs counted which contain 1 parasite is given by: (0.141/0.154) x 300 = 274.7

Therefore, the expected number of pRBCs containing >1 parasite per RBC (i.e. a multiply infected RBC) is 300 - 274.7 = 25.3

The observed number of multiply-infected RBCs is then divided by this expected number to generate a Selectivity Index (SI) value.

2.3.3. Mosquito Infection

On the day of a mosquito feed, a mouse was anaesthetised with an intra-muscular injection of 50µl Rompon / Vetalar and placed onto a pot of at least 30 female, 4-6 day old *Anopheles stephensi* mosquitoes. The mosquitoes were allowed to feed for 25 minutes before the mouse was removed and immediately euthanased whilst still under anaesthesia. Unfed mosquitoes were removed and the remainder maintained until dissection for mid-gut removal 8 days after the blood meal. Mid-guts were examined by light microscopy for the presence and number of oocysts in each mid-gut to indicate transmission success. As mosquitoes generally fed well and very few deaths occurred, most of the mosquitoes which were in each pot at the time of a blood meal survived to be dissected and examined for the presence of oocysts. The number of mosquitoes dissected was not normally distributed, so medians are given: 1) from each blood meal upon *P. c. adami*-infected CBA mice, a median of 28.5 mosquitoes was dissected (inter-quartile (IQ) range 25 to 30.25) and 2) from each blood meal upon *P. c. adami*-infected C57 mice, a median of 28 mosquitoes was dissected (IQ range 25 to 33). The prevalence of infection is shown as the percentage of mosquitoes infected out of the total number dissected. To test for the statistical significance of differences in infectivity to mosquitoes of DS and DK in the two mouse strains, Chi-squared (χ^2) tests were carried out (Minitab, version 14). To represent the oocyst burdens in the blood-fed mosquitoes, logarithms were taken of (the number of oocysts per dissected mosquito +1) and the arithmetic mean and standard error of these logarithms for each batch of blood-fed mosquitoes is given.

2.4. Results

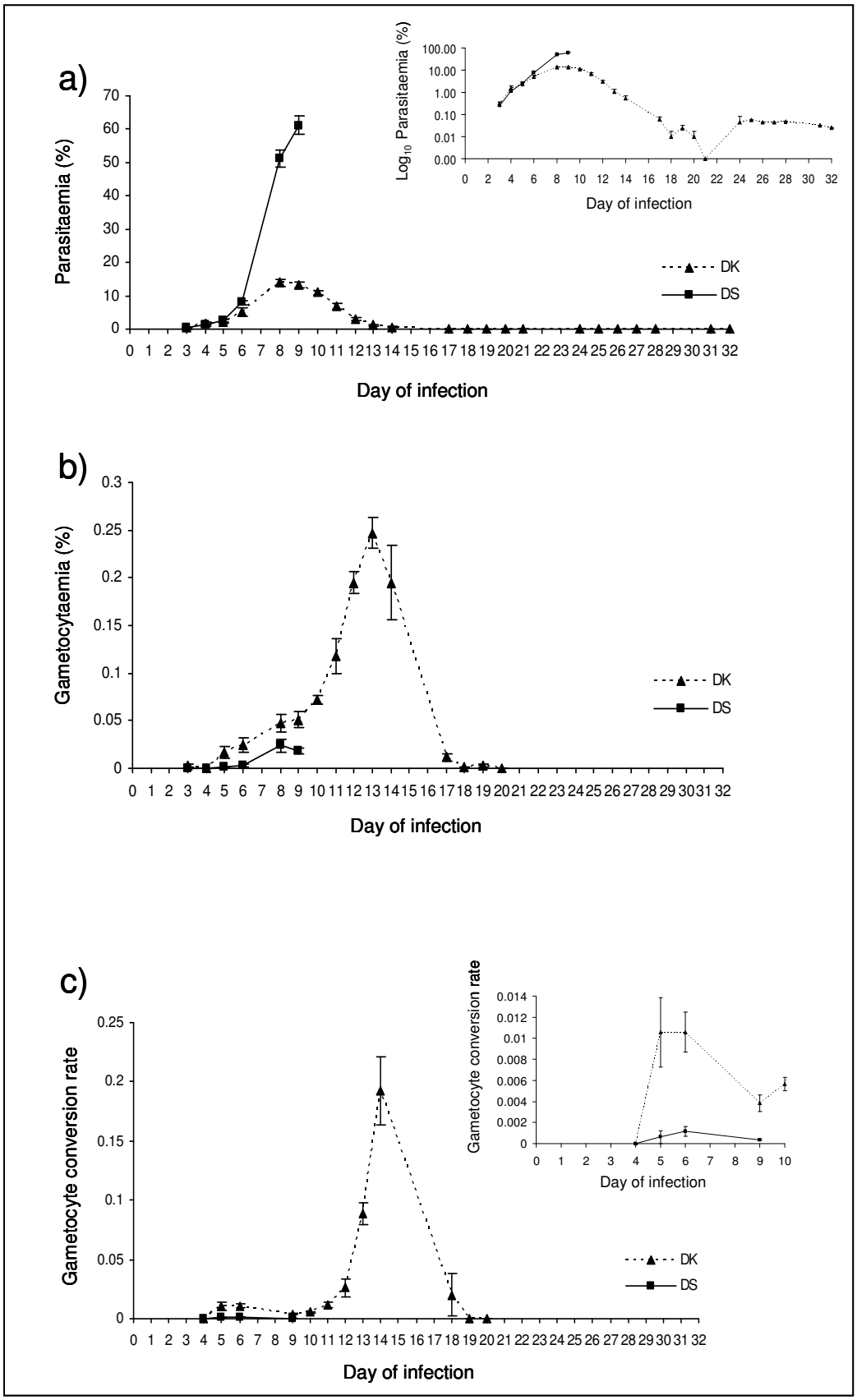
2.4.1. Asexual Parasite Growth and Gametocyte Production in *P. c. adami* Strains DS and DK in CBA Mice

Two groups of 5 female CBA mice were inoculated intra-peritoneally with 10^6 parasitised RBCs of either the DS strain or the DK strain of *P. c. adami.* Mice infected with DS reached parasitaemias of around 60% by day 9 post inoculation (at which point they were euthanased); those infected with DK peaked at around 14% on days 8 and 9 (Figure 2.1a). Up until day 6 post inoculation of the parasites, DS and DK parasitaemias were similarly low. Thereafter, parasitaemias in mice infected with DS continued to rise sharply until termination by euthanasia on day 9, while those in mice infected with DK declined after day 9 to undetectable levels by day 21. By day 24, two DK-infected mice again showed blood parasites at a very low level (Figure 2.1a inset), and this recrudescence continued in one mouse until the experiment was terminated on day 32.

Gametocytes were produced during DK infections on all days on which such recordings were made from day 3 to day 20, their numbers peaking on day 13 (Figure 2.1b), which was several days after the peak of asexual parasitaemia had occurred (Figure 2.1a). DK gametocyte conversion rates peaked on day 14 when the asexual parasitaemia was low (cf. Figure 2.1c with Figure 2.1a). DS infections produced significantly fewer gametocytes up to day 9 of infection than did DK (Student's *t*-test $p < 0.01$), in spite of having a much higher production of asexual parasites over this period (see Figure 2.1a). This was also reflected in the gametocyte conversion rates which were much lower for DS than for DK over the same period (Figure 2.1c inset).

Figure 2.1

Mice inoculated either with *Plasmodium chabaudi adami* DS (squares) or DK (triangles) were monitored for asexual stage parasitaemia (a) and gametocytaemia (b) over the course of infection. The gametocyte conversion rate (c) is the gametocytaemia on a given day (day n) divided by the asexual stage parasitaemia on the previous day (day n-1). Data given is the mean of 5 mice per group with error bars indicating the standard error of the mean.



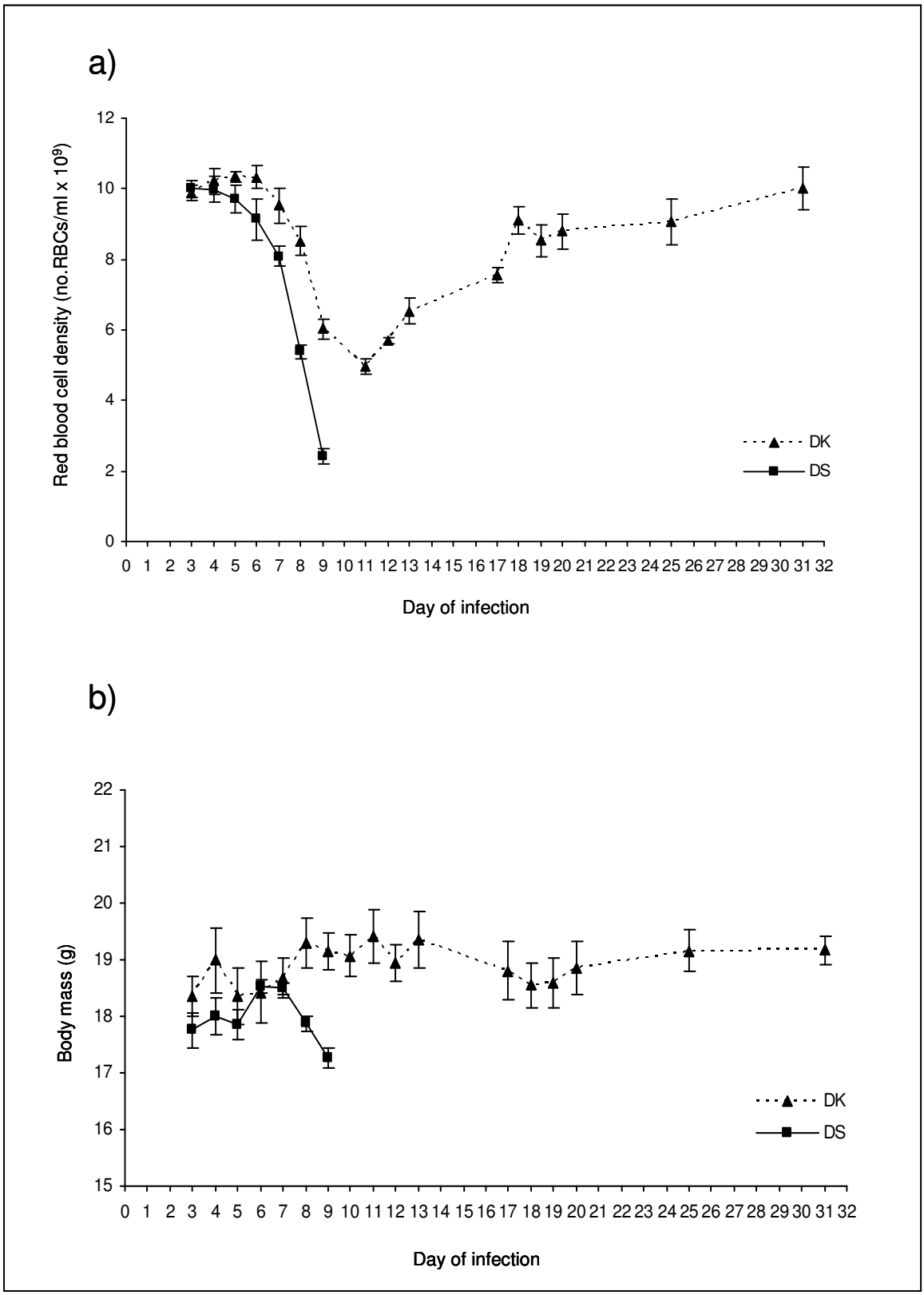
2.4.2. Pathogenesis of *P. c. adami* Infections in CBA Mice

By days 8 and 9, when DS infections had significantly higher parasitaemias than DK, the DS infected mice had lower RBC counts and body weights than did those infected with DK (Student's *t*-test $p < 0.01$) (Figures 2.2a and 2.2b). All mice infected with DS were showing signs of illness by day 9 and were euthanased. Mice infected with DK all survived until the experiment was terminated on the 32nd day of infection.

2.4.3. Selectivity of Invasion

One possible explanation for the lower maximum parasitaemia of DK relative to DS could be that a smaller proportion of the host RBC population is available for invasion by DK. To investigate this, the RBC Selectivity Indices (SI) of DS and DK blood parasites were measured at several time points during infections in order to determine if invasion was biased towards a subset of RBCs. If the SI=1, then the recorded numbers of multiply-infected and singly-infected RBCs are no different than would be expected if every RBC has an equal probability of being invaded. An SI >1 arises when there is a higher proportion of multiply-infected RBCs than would be expected if the RBCs were being invaded randomly, i.e. there is a subset of RBCs that is being preferentially invaded by the parasites. As shown in Figure 2.3, DS had a mean SI of around 1 on all days of measurement (between days 5 and 9 of infection) indicating unrestricted or random invasion of RBCs. The SIs measured for DK over the equivalent period were, by contrast, between 1.5 – 2, and thus almost twice those of DS; they did not change when the parasitaemias of DS and DK

Figure 2.2
Mean RBC densities as the number of RBCs/ml (a) and body weights (b) for mice inoculated either with *Plasmodium chabaudi adami* DS (squares) or DK (triangles) were measured over the course of infection. Data given is the mean of 5 mice per group with error bars indicating the standard error of the mean.



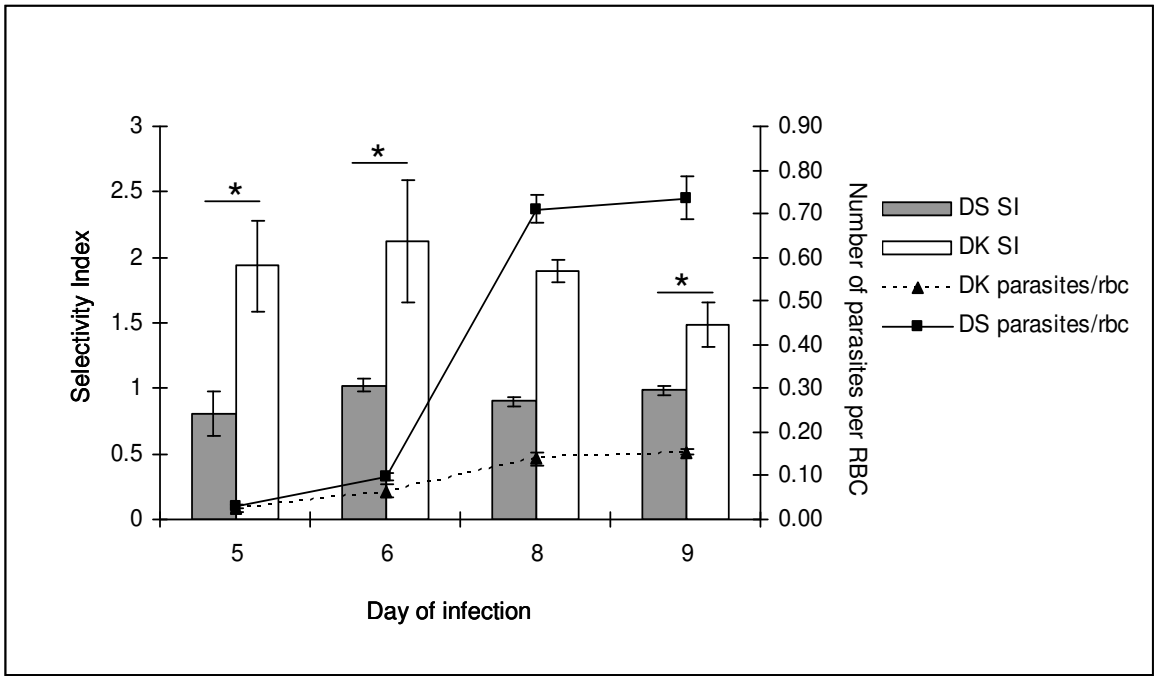
diverged after the 6th day of the infections (Figure 2.3). For DK, therefore, multiply-infected RBCs were around twice as likely to be found as would be expected by chance alone, suggesting that a subset of RBCs was being preferentially invaded by parasites of this strain. There was, however, no significant difference on any of the 4 days tested (5-9), between the percentage of reticulocytes infected with DK and the percentage of normocytes infected with DK (data not shown). This indicates that the selectivity of RBC invasion measured in DK infections was not due to a preference for reticulocytes as is known to occur in some malaria parasites.

2.4.4. Infectivity to Mosquitoes of Blood Infections of *P. c. adami* DS and DK in Mouse Strains CBA and C57

The infectivity of DS and DK to mosquitoes was investigated during blood infections of these parasites in two different mouse strains, CBA and C57, as two separate experimental series.

In the first experimental series, two groups of 10 female CBA mice were infected with 10^6 parasitised RBCs of either DS or DK. On days 3, 5, 6 and 9 after initiation of the infections, batches of at least 30 female *Anopheles stephensi* mosquitoes were allowed to blood feed on two mice from each group (DS or DK infected), thus giving two separate data points for infectivity to mosquitoes for each group for each day. There were no surviving mice infected with DS after day 9. However, mice infected with DK survived beyond this time and mosquitoes were blood-fed upon these mice on day 13 of their infections.

Figure 2.3
The Selectivity Indices of mice infected with *Plasmodium chabaudi adami* DS (dark bars) and DK (light bars) were compared on four different days of infection. The Selectivity Index is the number of multiply-infected RBCs observed, divided by the number of multiply-infected RBCs expected by chance, given the parasite burden. * indicates Student's *t*-test $p<0.05$ for a significant difference between DS and DK. Comparison by Student's *t*-test was not possible on day 8 since data were not normally distributed. Parasite burden on each day is shown as the number of parasites per red blood cell, DS (squares) or DK (triangles). Data given is the mean of 5 mice per group with error bars indicating the standard error of the mean.



DS and DK parasitaemias and gametocytaemias in this experimental series in CBA mice were comparable to those in the previous infections in CBA mice (cf. Figures 2.4a and 2.4b with Figures 2.1a and 2.1b). From the range of days examined, gametocytes were present in DS infections only on days 6 and 9 whereas DK produced gametocytes on days, 5, 6, 9 and 13, with the highest numbers being recorded on day 13.

In spite of gametocyte numbers being highest on day 13 of DK infections in CBA mice, their infectivity to mosquitoes, which was tested on days 3, 5, 6, 9 and 13, was highest on day 6 when the gametocyte numbers were considerably lower than on day 13 (Figures 2.4b, 2.4c and 2.4d). Day 6 was also the only day upon which mosquitoes became infected from the DS infections in CBA mice (Figures 2.4c and 2.4d), even though gametocyte numbers were higher in these infections on day 9 (Figures 2.4b). Thus, for both strains of *P. c. adami* in CBA mice, the optimum day of transmission to mosquitoes from within the range of days investigated was day 6.

Over the course of infection, both mosquito infection rates and oocyst densities in the infected mosquitoes were consistently higher in DK than in DS infections (Figures 2.4c and 2.4d). Table 2.1 shows that, even when considered only up until the 9^{th} day of infection (which is the time to which the DS infected CBA mice survived), transmission success was still significantly much greater for DK, the less pathogenic strain, compared to DS, the more pathogenic strain.

Figure 2.4
Transmission of *Plasmodium chabaudi adami* to mosquitoes from CBA mice was measured using two DS-infected mice (DS1, DS2; black/dark grey bars) and two DK-infected mice (DK1, DK2; light grey/white bars) on each of five time points during infection. The parasitaemia (a) and gametocytaemia (b) were measured for each mouse on the day of the blood meal along with the resulting percentage of mosquitoes infected (c) and the arithmetic mean $\log_{10}$ (oocyst burden +1) for all dissected mosquitoes feeding on a single mouse (d). Graph axes are the same as in Figure 2.5 to enable direct comparison. Error bars indicate the standard error of the mean.

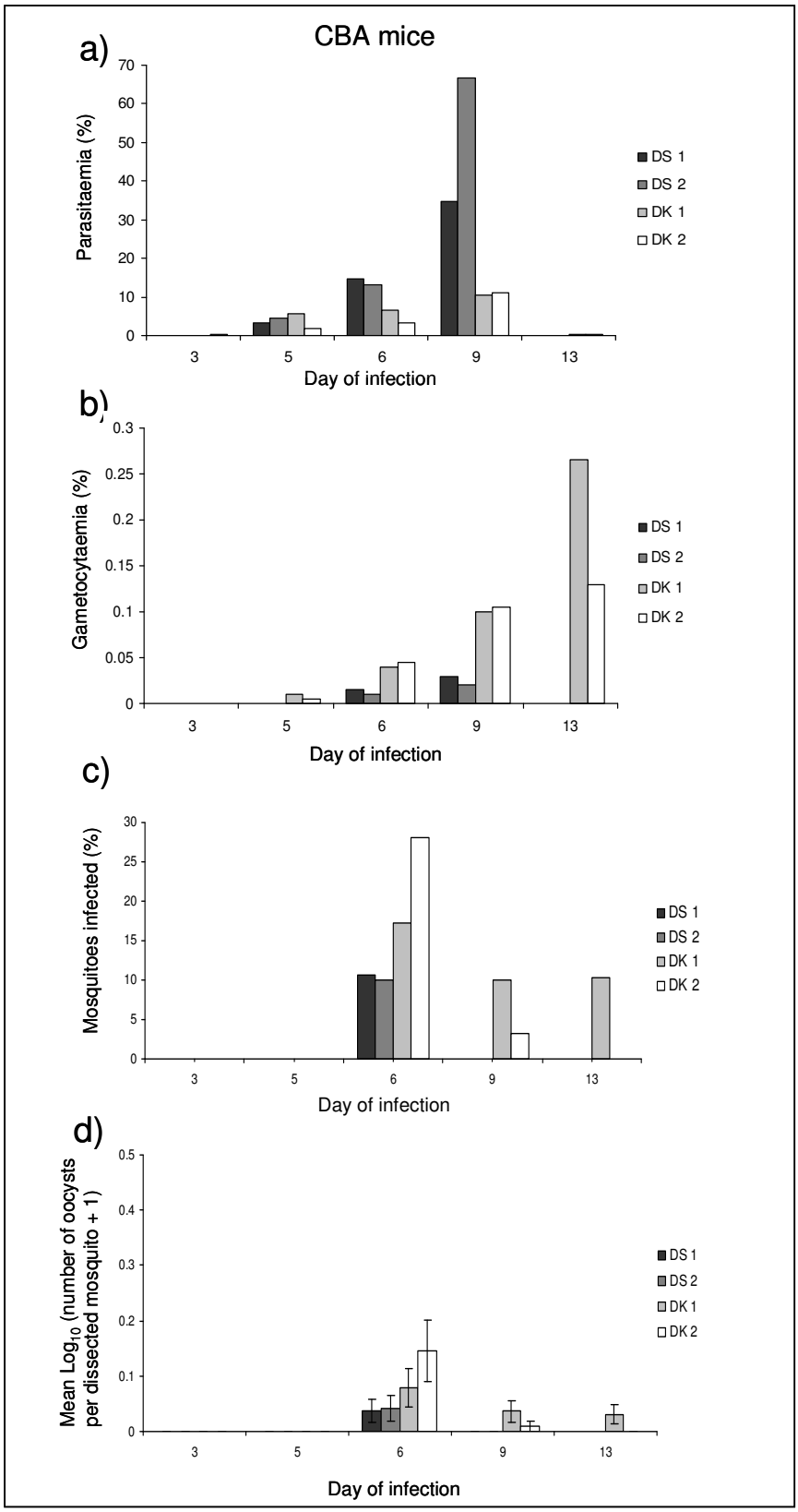


Table 2.1.
Infectivity of *Plasmodium chabaudi adami* strains DS and DK to mosquitoes (infected versus uninfected) on days 3, 5, 6 and 9 of blood infections in CBA mice, and on days 2 to 9, inclusive, of blood infections in C57 mice. For each combination of parasite and mouse strain, the data are the combined totals for all of the days on which batches of mosquitoes were fed (see Materials and Methods). Data were tested for significant differences between strains using the Chi-squared (χ^2) test.

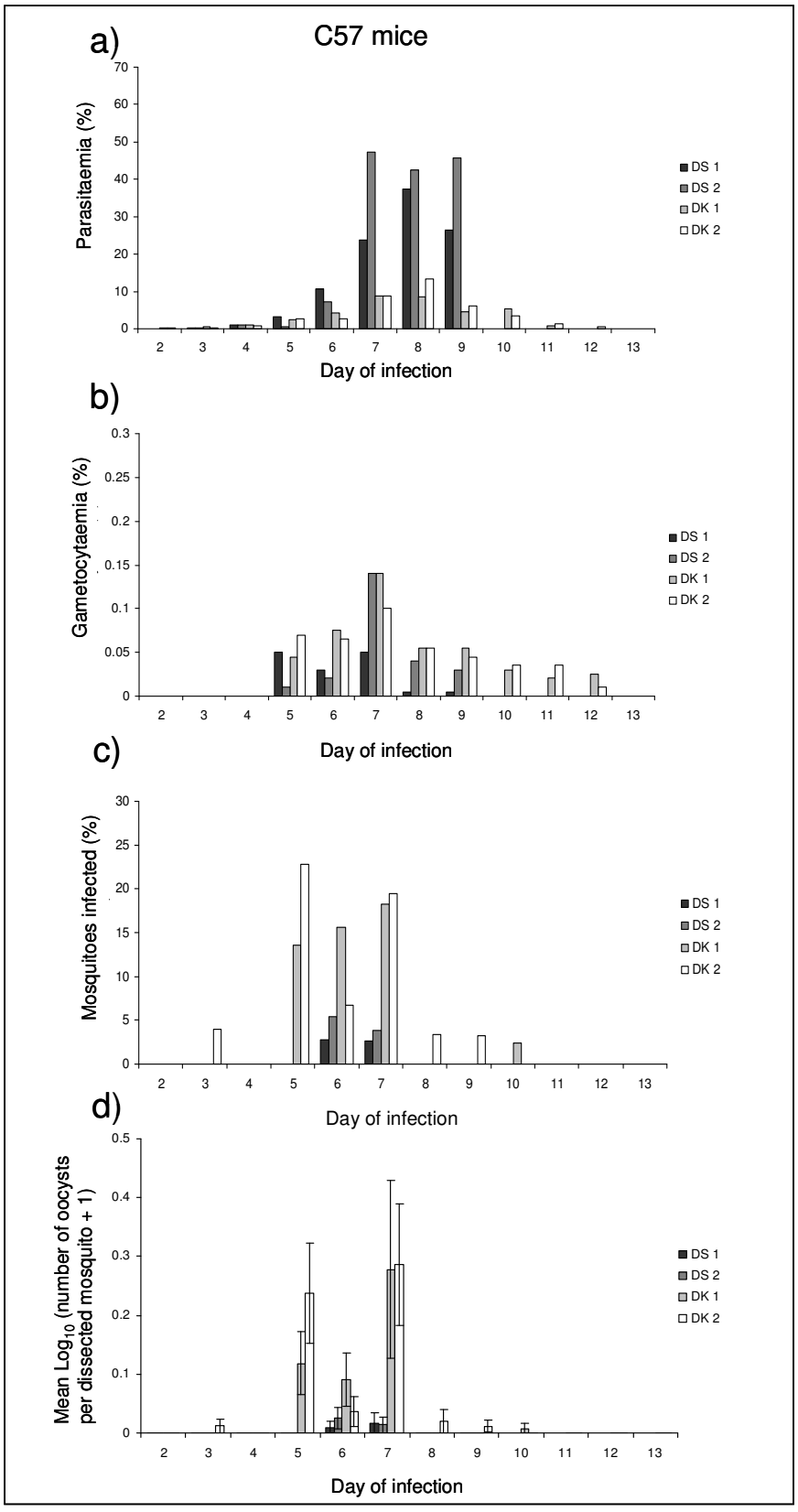
	Using mouse strain CBA		**Using mouse strain C57**	
	Mosquitoes		**Mosquitoes**	
Parasite strain	**Infected**	**Uninfected**	**Infected**	**Uninfected**
DS	6	218	5	410
DK	19	200	35	404
Mosquito infection rates for DS relative to DK	0.290		0.141	
χ^2	7.480, df = 1		21.887, df = 1	
p value	p<0.01		p<0.01	

This experimental protocol was repeated using C57 mice, and this time we tested for infectivity to mosquitoes on a daily basis from the second day of infection onwards. Two groups, each of 24 female C57 mice, were inoculated with 10^6 parasitised RBCs of either DS or DK. On each day, between days 2-13 after initiation of the blood infections, batches of at least 30 mosquitoes were allowed to blood feed upon two of the surviving mice from each group.

The courses of the asexual blood infections of DS and DK in the C57 mice (Figure 2.5a) were similar to those seen previously in the CBA mice (Figures 2.1a and 2.4a). Thus, as in the CBA mice, C57 mice infected with DS experienced much higher parasitaemias than did mice infected with DK, and the DS-infected mice were allowed to survive only until day 9 due to their high parasitaemias. DS produced similar numbers of gametocytes to DK over days 5-9, and for both strains, gametocyte numbers peaked on day 7 of infection (Figure 2.5b). In the DK-infected C57 mice, gametocyte numbers declined from day 8 onwards and ceased to be detectible on day 13 (Figure 2.5c). This is in striking contrast to the behaviour of DK in CBA mice, in which the highest gametocyte numbers were recorded on day 13 of infection (Figures 2.1b and 2.4b).

In C57 mice, the earliest that mosquitoes became infected with DK was day 3 and the latest was day 10 of infection. This is in contrast to the behaviour of DK in CBA mice, in which transmission did not occur on days 3 and 5 of infection, but did occur on day 13 (cf. Figures 2.5c and 2.5d with Figures 2.4c and 2.4d). DK was most infectious to mosquitoes from day 5 to day 7, comparable to its behaviour in CBA

Figure 2.5
Transmission of *Plasmodium chabaudi adami* to mosquitoes from C57 mice was measured using two DS-infected mice (DS1, DS2; black/dark grey bars) and two DK-infected mice (DK1, DK2; light grey/white bars) between days 2 and 13 post-infection. The parasitaemia (a) and gametocytaemia (b) were measured for each mouse on the day of the blood meal along with the resulting percentage of mosquitoes infected (c) and the arithmetic mean $\log_{10}$ (oocyst burden +1) for all dissected mosquitoes feeding on a single mouse (d). Graph axes are the same as in Figure 2.4 to enable direct comparison. Error bars indicate the standard error of the mean.



mice (cf. Figures 2.5c and 2.5d with Figures 2.4c and 2.4d). As had also been seen with infections in the CBA mice, DS blood infections in C57 mice had very limited transmission success, mosquitoes becoming infected on only two days, 6 and 7 (Figure 2.5c and 2.5d). Moreover, when measurements of infectivity of DS and DK to mosquitoes were limited to the period up until the 9^{th} day of infection (the period during which the mice infected with the more pathogenic strain, DS, survived) the transmission success of the more pathogenic DS was, again, significantly much lower than that of the less pathogenic DK (Table 2.1).

2.5. Discussion

This study has described the differential growth characteristics, pathogenicity, invasion selectivity and transmission success of two strains, DS and DK, of the rodent malaria parasite *P. c. adami*.

Mice infected with the *P. c. adami* DS strain experienced significantly higher peak parasitaemias than those infected with the DK strain, although both peaked around the same time, 8 to 9 days after the initiation of a blood infection with an inoculum of 10^6 parasites. This is in contrast to strains of the related sub-species *P. c. chabaudi*, which tend to peak by day 6 or 7 of a blood infection, given the same 10^6 parasite dose (Mackinnon and Read 1999; Timms et al. 2001). DS and DK did not differ in growth until day 6, therefore, for the optimum selection of DS-DK cross progeny for fast growth for LGS analysis, a pool of 10^6 DS x DK cross progeny must grow in the host for more than 6 days, preferably until the infection reaches a maximum parasitaemia similar to that of DS by day 8 or 9. DS was also more

pathogenic than DK, causing significantly more weight loss and anaemia. Therefore, these characteristics of DS and DK are in accordance with the positive association of maximum parasitaemia and pathogenicity seen in strains of *P. c. chabaudi* (Mackinnon and Read 1999; Mackinnon and Read 2004).

The slower-growing and less pathogenic DK was consistently more selective in its invasion of RBCs over the course of infection than the faster-growing and more pathogenic DS. Therefore, DK appeared to be restricted to the invasion of a sub-population of red blood cells. Despite the invasion selectivity of DK, there was no evidence for any invasion preference for reticulocytes over normocytes, confirming previous observations of *P. chabaudi* infections (Carter and Walliker 1976; Jarra and Brown 1989). There was also no significant change in either DK or DS invasion selectivity over the course of infection which might account for the difference in parasite growth occurring around day 6 of infection. Specifically, DK did not become more or less selective over time, indicating that the proportion of RBCs available for DK invasion was similar over the course of the infection. In a mathematical model of the dynamics of infection with *P. c. chabaudi* strains AS and AJ, differences in peak parasitaemia between the two strains were attributed to their different red blood cell age preferences for invasion (Antia et al. 2008). AJ, which reached a higher parasitaemia than AS, was thought to invade a wider red blood cell age-range, however the actual invasion selectivity of these two strains was not measured. Therefore, although DK was more selective than DS and was restricted to a subpopulation of normocytes, which might be of a particular age, the exact

contribution of this factor to the difference in growth between these two strains is unclear.

Strains of *P. c. chabaudi* that have higher maximum parasitaemias and higher pathogenicity have been reported to have greater gametocyte production and higher infectivity to mosquitoes (Ferguson et al. 2003; Mackinnon and Read 2004). This was not the case, however, in the present study of pathogenic and non-pathogenic strains of *P. c. adami*.

DS gametocyte production in CBA mice was significantly lower than that of DK, despite DS producing significantly more asexual parasites. The pattern of DK gametocyte production was strikingly different between two mouse strains; DK infections produced most gametocytes before the peak of parasitaemia in C57 mice, whereas in CBA mice, DK infections produced the largest numbers of gametocytes after the peak of parasitaemia. These dynamics of gametocyte production are different to those of strains of *P. c. chabaudi* in C57 mice, in which gametocyte production peaks several days after the peak of parasitaemia (Mackinnon and Read 1999; Ferguson et al. 2003; Mackinnon and Read 2003). Therefore, the dynamics of gametocyte production are influenced by the genetic constitution (whether strain or subspecies) of both parasite and host.

In both host strains however, the faster-growing, pathogenic DS transmitted significantly less well than the slower-growing, less pathogenic DK. Moreover, the relationship between the timing of maximum gametocyte production and the day of

peak infectivity to mosquitoes was not straightforward. For both parasite strains in both hosts, infectivity to mosquitoes was highest just before the peak of asexual parasite production, around the 5th to 7th days of infection, regardless of differences in the timing of gametocyte production between host strains. Infectivity of strains of the *P. c. chabaudi* subspecies was examined between the 12th and 15th days of infection, which is after the peak of asexual parasite production and around the time of maximum gametocyte production in these parasites (Mackinnon and Read 1999; Ferguson et al. 2003). Therefore, although *P. c. adami* and *P. c. chabaudi* are closely related, there are clearly genetic differences between the two subspecies which influence transmission. Subsequently, even within the same parasite species, no general rule can predict the relationship between growth / pathogenicity and infectivity to mosquitoes.

Furthermore, infections of *P. cynomolgi*, *P. gallinaceum*, *P. yoelii*, and *P. berghei,* are often most infectious to mosquitoes before the peak of parasitaemia, and generally at a time before gametocytes are at maximum densities (Carter and Gwadz 1980; Dearsly et al. 1990; Naotunne et al. 1990). Infectivity to mosquitoes may not correlate with gametocyte production at a particular point in the infection because of many factors; for example, the changing maturity or male/female sex ratio of gametocytes over time, or in particular, the presence of cytokines or antibodies which have inhibitory or enhancing properties (Carter and Gwadz 1980; Carter and Graves 1988; Naotunne et al. 1990; Naotunne et al. 1991; Karunaweera et al. 1992).

Therefore, when transmitting DS and DK to mosquitoes for the purposes of generating genetic crosses, it would appear that the day of infection is the most important factor influencing transmission success. In the light of the results from single DS and DK infections, days 6 or 7 of infection are potentially the best days to transmit to mosquitoes. Higher oocyst burdens seemed to be more likely to be achieved by using C57 mice rather than CBA mice, but overall DS was a poorer transmitter than DK. In order to generate the maximum proportion of recombinants from the meiotic products of a cross, the mosquito must take up equal numbers of infectious gametocytes from each parent. Given that DS is less infectious, it might be beneficial to reduce the amount of DK relative to DS in the mixed infection, to maximise the efficiency of DS x DK recombination. Whether the transmission characteristics of DS and DK from single strain infections will reflect the nature of their transmission from mixed infections will not be apparent until the first crosses are undertaken, but this information is a good starting point.

Chapter 3: *P. c. adami* DS and DK Growth in Mixed Infections

3.1. Foreword

The individual growth characteristics of DS and DK, which are the basis for the phenotype under selection in the present genetic analysis, have been examined in Chapter 2. However, there are points during the LGS process where DS and DK are present together in the same host and could, therefore, influence each other's growth. Firstly, when a mixed DS-DK infection is generated and grown over 6 or 7 days in the blood of a mouse before transmission to mosquitoes for LGS, transmission of these individual strains may be influenced by interactions between them. Knowledge of how DS and DK might influence each other's growth in a mixed infection, is useful in determining what ratio of DS:DK to use in a mixed infection for optimum transmission of both strains on a particular day.

Secondly, when the cross progeny pool is grown up in mice over several days to select for fast growth, it will consist not only of DS x DK recombinants, but DS and DK parental-type parasites as well. Although most of the slow-growing DK parental parasites should be eliminated by the final round of fast growth selection, there is still a potential for the DS and DK parentals to affect the growth of the recombinant progeny, as well as each other. Since the growth characteristic of the cross progeny pool is the phenotype under investigation, it is important to understand which factors might influence this.

Therefore, the following study was set up to examine the effect of DS and DK on each other's growth in simple mixed infections, and how this might be modulated by changing the ratio of DS:DK in the starting inoculum. Unequal co-infections of this sort are the most likely result of the inoculation of cross progeny for blood stage growth selection in an LGS investigation. In a similar way to the results from Chapter 2, it was clear that the results of the study were not only of use for the LGS investigation, but were also of interest within the context of the existing literature on mixed infections of malaria parasites. Therefore, this chapter also examines the broader implications of the findings, as well as those of relevance to the objective of this thesis.

3.2. Introduction

Mixed species infections of *Plasmodium* in human and non-human hosts are common in nature (Richie 1988; Bruce et al. 2000; Snounou and White 2004). The possibility that co-infecting human malaria parasites may interact with each other is raised by observations of the exclusion of one parasite species by another, or the sequential appearance of one species after another, in the same host over a period of time (Richie 1988; Bruce et al. 2000; Snounou and White 2004). Testing the nature and outcome of these interactions in natural human malaria infections is difficult because of other confounding factors, therefore, experimental studies using rodent malaria parasites in the laboratory have also been undertaken. A number of wild-caught thicket rats from Central Africa were found to harbour more than one species of rodent malaria parasite (Carter and Walliker 1975, 1976) and as such, the natural

population of rodent malaria parasites may also exist in both single and mixed infections.

Many experimental rodent malaria co-infection studies have looked at mixed infections resulting from the simultaneous inoculation of equal numbers of two or more different parasite strains or species (Snounou et al. 1992; DeRoode et al. 2004; DeRoode et al. 2005a; DeRoode et al. 2005b; Bell et al. 2006; Raberg et al. 2006; Wargo et al. 2007). All demonstrate some effect on growth, of either or both strains, of being in a mixed infection. The nature and direction of these effects were dependant on the particular co-infecting strains or species, although at least for strains of *P. c. chabaudi*, it has been reported that more pathogenic strains were capable of suppressing the growth of less pathogenic strains (DeRoode et al. 2005b; Bell et al. 2006).

Some studies have examined mixed infections generated by super-infection, i.e. where a host already infected with one type of malaria parasite is later infected with another. A number of experimental super-infections in primates and in humans during malaria-therapy for neurosyphilis, showed some degree of suppression of the growth of one species by another which depended on the relative timing of the infections (Richie 1988). In rodent malaria infections initiated with equal numbers of parasites of different strains or species given several days apart, the timing of the super-infection, as well as the species or strain of parasite, was important in influencing the outcome of infection (Richie 1988; Snounou et al. 1989; Read et al. 2002; DeRoode et al. 2005a; Voza et al. 2005). However, in some cases with

particular parasite genotypes, inoculating a less pathogenic strain or species in advance adversely affected the growth or pathogenicity of a super-infecting, more pathogenic strain or species (Snounou et al. 1989; Read and Taylor 2001; DeRoode et al. 2005a; Voza et al. 2005).

One important aspect to consider is the effect that an unequal starting ratio of co-infecting parasites might have on their subsequent growth in the mixed infection, particularly if these parasites differ in pathogenicity. Co-infections in nature are unlikely to always result from equal inocula of different parasite strains, because the mosquito may have originally taken up different numbers of gametocytes of different parasite strains or species present in the same host (Nwakanma et al. 2008).

A number of studies where mixed infections were initiated with inocula containing different ratios of the co-infecting strains have examined differences in pathogenicity, transmission and total parasite density between mixed and single infections (Taylor et al. 1997; Taylor et al. 1998; Taylor and Read 1998; DeRoode et al. 2003). However, only one study has measured the growth of each co-infecting rodent malaria strain in mixed infections of different starting ratios, and compared it with the growth of the same strain, at the same inoculum size, in a single infection (Taylor et al. 1997). In this case, however, the parasite strains used did not differ in pathogenicity. Since the rodent malaria parasite strains to be used in the present LGS analysis do differ significantly in pathogenicity, it is important to examine the possible interaction of pathogenic and non-pathogenic strains in unequal co-infections. In this study, mixed infections were initiated with a range of inoculum

ratios of the fast-growing and pathogenic *P. c. adami* DS strain and the slow-growing and non-pathogenic *P. c. adami* DK strain, and growth was compared to those of single infection controls.

3.3. Materials and Methods

3.3.1. Parasites

The rodent malaria parasite strains *P. c. adami* DS and DK used in these experiments have already been described in detail in section 2.3.1 of Chapter 2.

3.3.2. Single-Strain and Mixed-Strain Infections

Fifty-three female CBA mice (6-8 weeks old) were infected with either DS, DK, or a mixture of the two. Three groups of five mice were infected with different sized inocula of DS, three groups of five mice were infected with the equivalent inocula of DK, and the remainder with mixtures of different starting inoculum ratios of DS:DK, as outlined in Table 3.1.

To generate a single strain infection, a small volume of donor mouse blood containing the calculated parasite inoculum (either $5x10^5$, $5x10^4$ or $5x10^3$ DS or DK parasitised red blood cells (pRBC)) was added to 0.1ml 50% Fetal Calf Serum 50% Ringers solution and injected inter-peritoneally into a recipient mouse. To generate a mixed DS/DK infection, the required numbers of pRBC from each strain were mixed together in 0.1ml 50% Fetal Calf Serum 50% Ringers solution and this mixture was

Table 3.1
The number of mice in each group infected with different starting amounts of *Plasmodium chabaudi adami* DS or DK pRBCs is given, along with the DS:DK ratios of these amounts and the final day of infection for each group.

Starting inoculum (per mouse)			**Number of mice**			**Final day of infection**
Number of pRBC of each strain	**Total number pRBC**	**Ratio DS:DK**	**Inoculated on day 0**	**Used in analysis**	**On final day**	
$5x10^3$ DS	$5x10^3$	1:0	5	5	5	12
$5x10^4$ DS	$5x10^4$	1:0	5	4	4	11
$5x10^5$ DS	$5x10^5$	1:0	5	5	2	9
$5x10^3$ DK	$5x10^3$	0:1	5	5	5	14
$5x10^4$ DK	$5x10^4$	0:1	5	5	5	14
$5x10^5$ DK	$5x10^5$	0:1	5	5	5	14
$5x10^5$ DS + $5x10^3$ DK	$5.05x10^5$	100:1	4	4	3	9
$5x10^5$ DS + $5x10^4$ DK	$5.50x10^5$	10:1	4	3	3	9
$5x10^5$ DS + $5x10^5$ DK	$10x10^5$	1:1	5	4	2	10
$5x10^4$ DS + $5x10^5$ DK	$5.50x10^5$	1:10	5	5	5	10
$5x10^3$ DS + $5x10^5$ DK	$5.05x10^5$	1:100	5	5	5	10

then injected into the recipient mouse; therefore both strains were injected simultaneously. Blood smears (one per mouse per day) were made with a thin smear of tail blood on a microscope slide, fixed with methanol and stained with a solution of 10% Giemsa's stain in PBS pH 7.2 for 30 minutes. Approximately 1,000 red blood cells in five fields of vision per slide (at 1250x magnification under oil immersion) were examined for the percentage infected to give the parasitaemia. The mean and standard error of the mean (sem) were calculated for the mice in each group. Certain time-points were compared using the Student's *t*-test or one-way ANOVA and data were found to be normally distributed. Additionally, a small drop of tail blood was taken daily from each mouse in each mixed infection group and stored dried on FTA Classic Card (Whatman). DNA was later extracted from these blood spots for PCR and pyrosequencing.

3.3.3. DNA Extraction from Blood Samples and PCR

DNA was extracted from circles punched out of dried blood spots on the FTA Classic Card (one sample per mouse per day) using a 1.2mm diameter micro-punch (Harris) and washed in FTA Reagent (Whatman) and sterile distilled water as described in detail in Chapter 6, section 6.2. A short, biotinylated fragment of the *ama-1* gene containing a single nucleotide polymorphism (SNP) differentiating DS and DK was amplified directly from the sample disc by PCR. PCR was carried out according to the method of Cheesman and others (Cheesman et al. 2007) and as described in more detail in Chapter 5, section 5.2.3. Briefly, in a 50µl reaction volume made up with sterile MilliQ water, the following were mixed: 1x Immobuffer (Bioline), 1.5mM $MgCl_2$, 0.3mM dNTPs, 1 unit Immolase DNA polymerase

(Bioline) and 0.3µM *ama-1* forward and reverse primers (Eurogentec), and added to the sample disc. Forward and reverse primers were designed using Pyrosequencing™ Assay Design Software Version 1.0.6 (Biotage) and the sequences of the DS and DK *ama-1* genes. Primer sequences: forward *ama-1* biotinylated primer 5'-TGATATGTCATTGTGTGCAAAAC-3' and reverse *ama-1* primer 5'-TGGACCCATATTTTCTTGTGC-3'. The following reaction conditions were used to amplify the *ama-1* gene fragment to levels sufficient for pyrosequencing quantification: 7 minute 95°C initial activation step, then 38 cycles of [93°C 60 seconds, 64°C 60 seconds , 72°C 60 seconds], ending with a 10 minute 72˚C hold. PCR products were visualised under UV light on 1.5% agarose gels stained with Ethidium Bromide, to confirm successful amplification.

3.3.4. Quantification of DS and DK Proportions in Mixed Infections using Pyrosequencing

Pyrosequencing has been validated as an accurate technique for allele quantification in mixtures of *P. chabaudi* and the method used here is as described by Cheesman and others (Cheesman et al. 2007) and is described in more detail in Chapter 5. Briefly, in a 96-well plate format, 5µl of the biotinylated PCR product was added to 35µl Streptavidin-Sepharose high performance beads (Amersham Biosciences) in Binding Buffer solution (Biotage) and 40µl sterile distilled water, and the plate was shaken for 10 minutes. Beads with attached DNA were taken up from each well using the Pyrosequencing™ Vacuum Prep Tool (Biotage) and then washed sequentially with 70% ethanol, 0.2M sodium hydroxide, and wash buffer (Biotage) before being transferred into a 96-well pyrosequencing plate containing 0.5µM

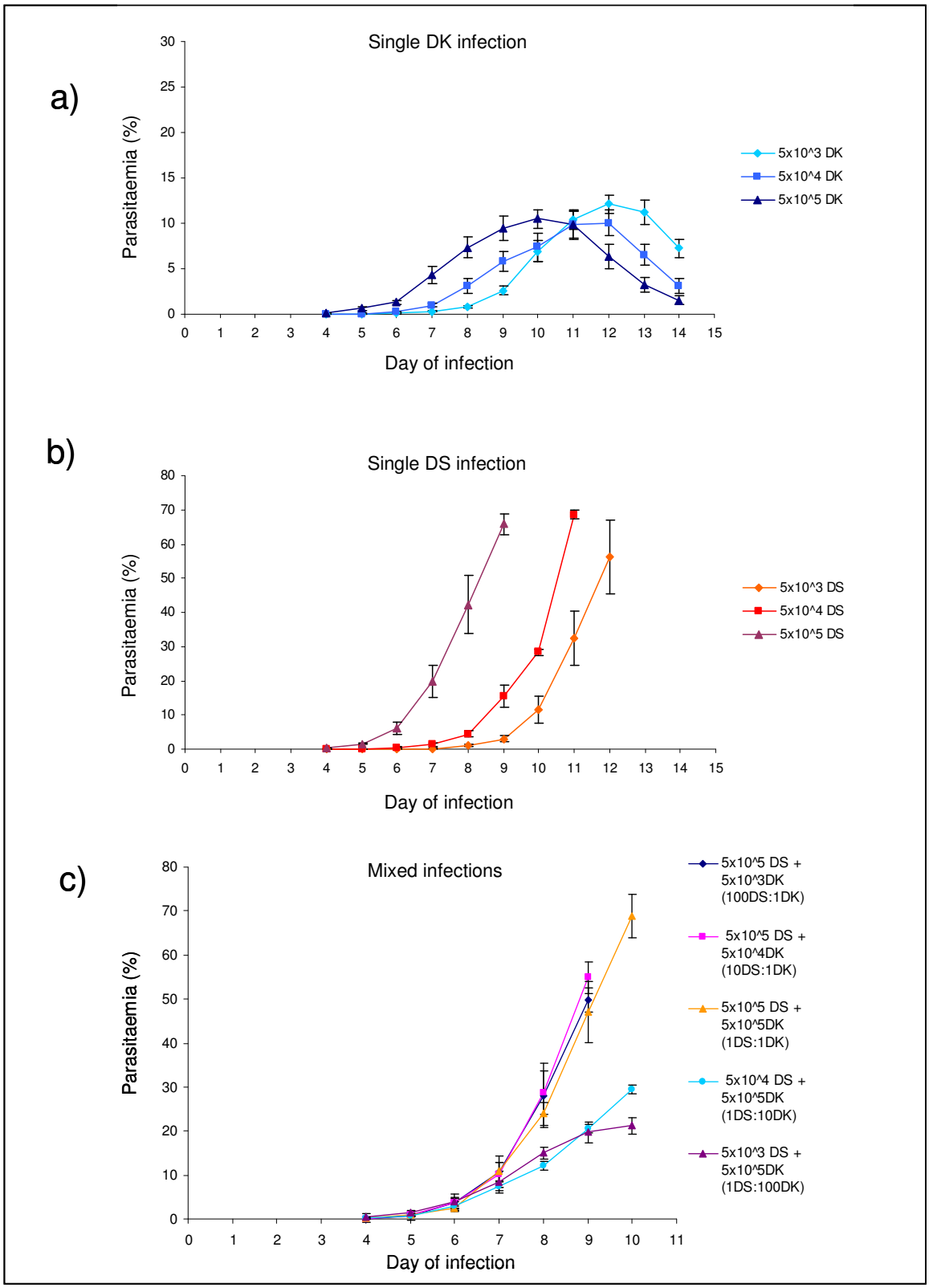
sequencing primer (Eurogentec) in annealing buffer (Biotage). The sequencing primer was: 5'-TCATAAACAGCGGGAT-3'. This plate was heated at 80˚C for 120 seconds, and once cooled to room temperature, analysed by the PSQ HS 96A Pyrosequencer instrument. Samples were run in duplicate or triplicate and only those passing automatic and manual quality control checks were included for analysis. The parasitaemia attributable to each individual strain at each time point was calculated from the proportion of each strain as determined by pyrosequencing and the total parasitaemia of the mixed infection as determined by microscopy. The mean and standard error of the mean (sem) were calculated for the mice in each group and certain time-points were compared using the Student's *t*-test; data were found to be normally distributed.

3.4. Results

3.4.1. Single Infection Parasitaemia

Groups of 5 female CBA mice were infected either DS or DK, in three different inoculum sizes and their infections were followed for 14 days (Figure 3.1). Single DK infections of $5x10^5$ pRBC led to a parasitaemia peaking on day 10 post-infection at 10.5% (Figure 3.1a). Decreasing the inoculum size 10-fold to $5x10^4$ pRBC resulted in a peak at 10% on days 11-12. Decreasing the inoculum size 100-fold to $5x10^3$ pRBC resulted in a peak at 12% on day 12. Therefore, 10-fold decreases in DK inoculum size had no effect on maximum parasitaemia (one-way ANOVA $F_{2,8}=0.63$ $p=0.428$) but resulted in approximately a one day delay in time to peak parasitaemia.

Figure 3.1
Mice inoculated with either with *Plasmodium chabaudi adami* DK (a), DS (b) or mixtures of DS and DK (c), were monitored for asexual stage parasitaemia over the course of infection. Single infections were initiated with either $5x10^5$, $5x10^4$ or $5x10^3$ pRBCs. Mixed infections were initiated with a range of inocula sizes of DS and DK. Data given is the mean of all mice in the group with error bars indicating the standard error of the mean.



In the $5x10^5$ pRBC DS single infection group, only two mice remained on day 9 with a peak parasitaemia of 66% (Figure 3.1b). The 3 other infections had been terminated on day 8 with parasitaemias in the range 40%-74%, as mice were euthanased at the first sign of illness. In the $5x10^4$ pRBC DS group, 1 out of the 5 mice developed a parasitaemia rapidly ascending to 80% on day 8 and was excluded from comparison with the others in the group. Of the 4 remaining, infections reached an average 69% parasitaemia on day 11. In the $5x10^3$ pRBC DS group, all five mice reached day 12 with an average parasitaemia of 56%. Again, the maximum parasitaemias of each group were not significantly different from each other (one-way ANOVA $F_{2,11}=0.67$ $p= 0.534$) but time to maximum parasitaemia was delayed by 1-2 days for every 10-fold decrease in inoculum size. Therefore, despite the different growth characteristics of DS and DK, the effect of inoculum size on time to maximum parasitaemia was similar for both strains.

3.4.2. Mixed Infection Parasitaemia

Mixed infections were followed for 10 days and total parasitaemia levels for the 5 mixed infections are shown in Figure 3.1c. The final day parasitaemias of the 1DS:10DK (light blue) and 1DS:100DK (purple) groups were lower than those of the 10DS:1DK (pink) and 100DS:1DK (dark blue) groups respectively, even though they started with the same total number of parasites (Figure 3.1c and Table 3.1). There were two unusual observations; one mouse in the 10DS:1DK group had only reached a parasitaemia of 4% by day 9 when the other mice had parasitaemias of approximately 55%, and one mouse in the 1DS:1DK group developed a much more rapidly ascending parasitaemia than the other mice, peaking on day 8 when the

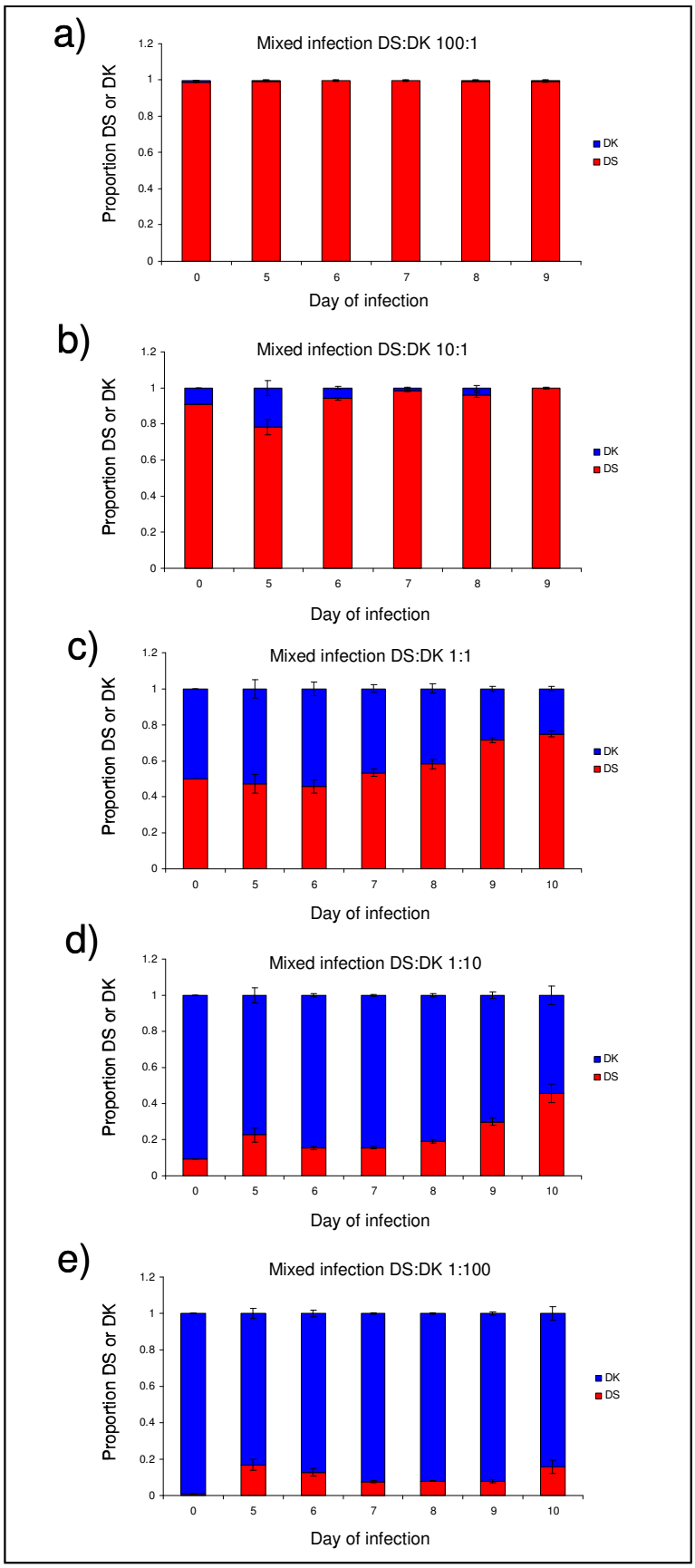
others had an average parasitaemia of 24%. On rare occasions, in the experience of this laboratory, a mouse within a group of mice given a particular parasite inoculation, may show an infection profile unlike the others in the group. This could be the result of insufficient mixing of the parasite inoculum before injection, or due to a particular mouse in a batch of littermates being significantly more or less susceptible to the infection, compared to the others. It was necessary to exclude the two cases observed here from further analysis, in order to make a true comparison between groups of apparently equally susceptible mice, intentionally given different inoculum sizes.

3.4.3. DS and DK Proportions in Mixed Infections

The analysis of the proportions of DS and DK in mixed infections by pyrosequencing was only possible from day 5 onwards due to low parasite numbers on the preceding days. DS retained its predominance over the course of infection when given an initial numerical advantage in the inoculum (Figures 3.2a and 3.2b). The proportion of the parasitaemia attributable to DS in the 100DS:1DK infection from day 5 onwards was always greater than 99% (Figure 3.2a). In the 10DS:1DK infection, 80% of the parasitaemia was DS on day 5, increasing over time to more than 99% (Figure 3.2b). At an initial 1DS:1DK inoculum (Figure 3.2c), the proportions of DS and DK were approximately equal on day 5 but then the proportion of DS increased, reaching 75% by day 10.

When DK was in the starting majority by 10-fold (1DS:10DK) in the inoculum (Figure 3.2d), it also remained predominant, although the proportion of DS increased

Figure 3.2
Mean proportions of *Plasmodium chabaudi adami* DS (red) or DK (blue) over time in mixed infections initiated with five different ratios of DS:DK in the inoculum (a-e). Data given is the mean of all mice in the group with error bars indicating the standard error of the mean.



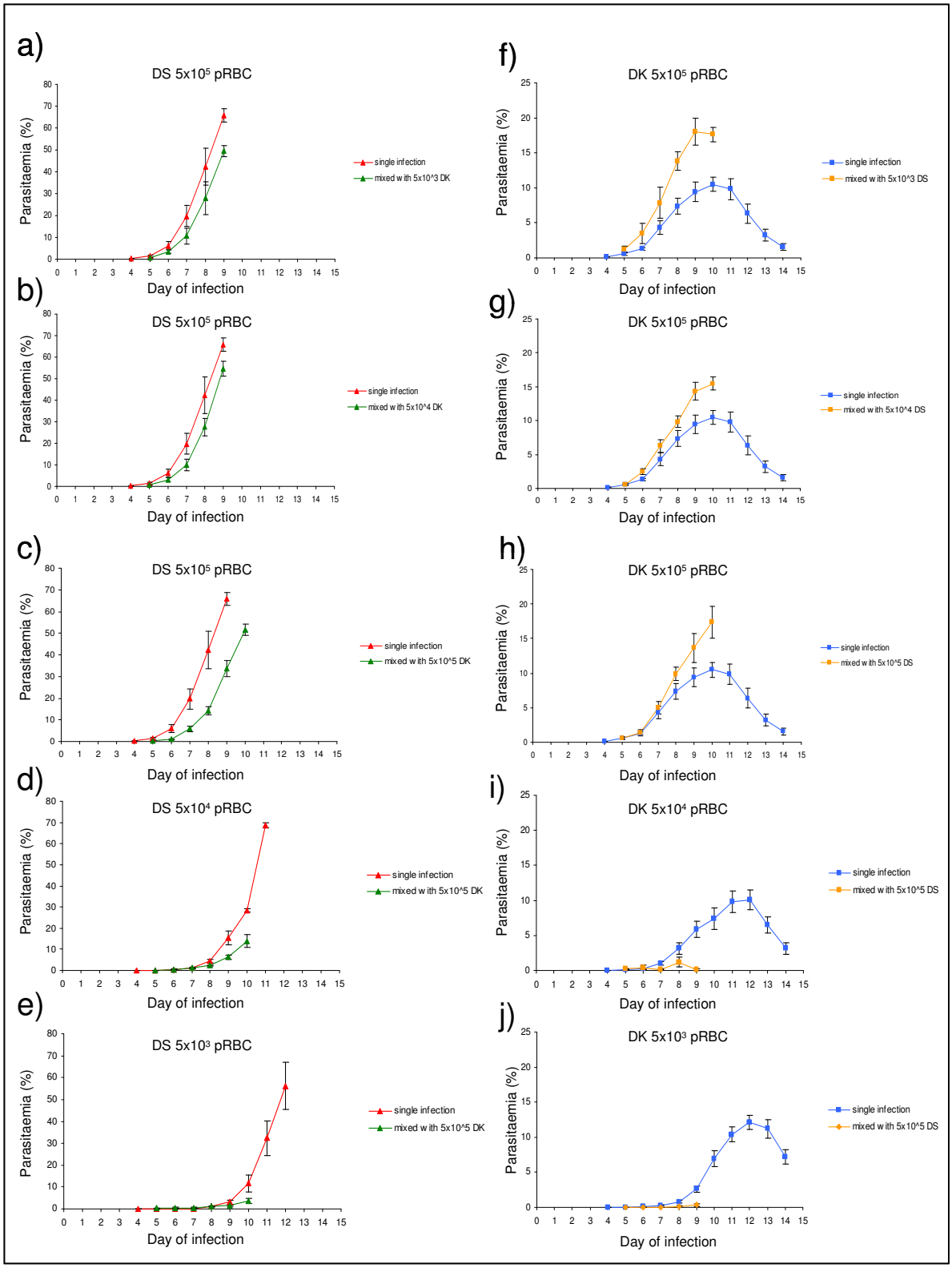
steadily and only 55% of the parasitaemia was still DK by day 10. When DK was initially 100-fold more than DS (1DS:100DK) (Figure 3.2e), the majority of the parasitaemia (85%) was still due to DK by day 10. The two infections which were still dominated by DK after 10 days of growth (1DS:100DK and 1DS:10DK) had lower overall parasitaemias than the three dominated by DS (Figure 3.1c).

3.4.4. DS and DK Growth in Single and Mixed Infections

The growth profiles of DS and DK in single and mixed infections of the same inoculum size is shown in Figure 3.3. Although DS was able to dominate a mixed infection when DK was present at an equal or lower level in the starting inoculum, DS grew to a lower parasitaemia in the presence of DK than it did in a single infection (Figure 3.3 a-e). In a final day of observation (day 9) parasitaemia comparison, DS parasitaemia in the 100DS:1DK infection group was significantly lower than in the single infection control (Student's *t*-test $p=0.03$) (Figure 3.3a) and there was also a non-significant trend in this direction in the 10DS:1DK infection group on day 9 (Student's *t*-test $p=0.114$) (Figure 3.3b). By day 9 of the 1DS:1DK mixed infection, DS parasitaemia was also significantly lower than in the single infection (Student's *t*-test $p<0.01$) (Figure 3.3c) and the addition of DK at this ratio appears to have delayed the growth of DS by a day. Therefore, an equal, or even a minority amount, of the slow-growing DK in the infection inoculum slowed the normal pattern of DS growth.

In the reverse experiment, when DK was in the majority or equal to DS (Figure 3.3f-h), DK reached a higher parasitaemia in a mixed infection with DS than it did alone.

Figure 3.3
Mean parasitaemias over the course of infections, for *Plasmodium chabaudi adami* DS in single (red) and mixed (green) infections (a-e) with DK, and DK in single (blue) and mixed (yellow) infections (f-j) with DS. Data given is the mean of all mice in the group with error bars indicating the standard error of the mean.



In all three of the 1DS:100DK, 1DS:10DK and 1DS:1DK infection groups, DK reached a significantly higher parasitaemia on day 10 than it did as a single strain infection (Student's *t*-test $p<0.01$, $p<0.01$ and $p=0.02$ respectively) (Figure 3.3f-h). Thus DK growth was actually increased, rather than reduced, by the presence of an equal or minority amount of DS in the starting inoculum.

When DK was in the majority compared to DS, DS growth was reduced relative to its growth at the same inoculum size in a single infection by day 10; 1DS:10DK Student's *t*-test $p<0.01$ (Figure 3.3d) and 1DS:100DK Student's *t*-test $p=0.07$ (Figure 3.3e). When DS was in the majority compared to DK, by 10 or 100-fold, DK growth by day 9 was always significantly reduced by the presence of DS (Student's *t*-test $p=0.01$) (Figure 3.3i and 3.3j). Therefore, both DS and DK were capable of reducing each other's growth when they were in the majority in the starting inoculum.

3.5. Discussion

This study has described the effect of initiating mixed infections with different ratios of two parasite strains DS and DK, on their subsequent growth relative to that in single infections. The slow-growing, non-pathogenic DK strain was found to be capable of suppressing the growth of the faster-growing, pathogenic DS strain to varying degrees. In contrast, in some infections, the presence of DS led to DK reaching higher parasitaemias in a mixed infection than it did on its own. Furthermore, the extent and type of effect that the two strains had on each other were dependent on the ratio of DK:DS in the starting inoculum. Whatever the starting ratios, the normal growth characteristics of both strains were always affected, in one

way or another, by growth in a mixed infection. This effect has been seen before in other rodent malaria co-infection studies where changes in mortality, maximum primary and recrudescent parasitaemia and growth to peak parasitaemia, were all observed (Snounou et al. 1992; DeRoode et al. 2005a) . However, these studies do not necessarily address the effect of differences in growth between co-infecting parasites, such as in the present study. As had been found in Chapter 2, single infections of DK were non-pathogenic, with peak parasitaemia only around 10% and the infections were quickly resolved. Single infections of DS rapidly grew to high parasitaemias, at which point the infections were terminated due to the pathogenicity of the infection. However, the relative pathogenic effects of the two strains were not altered by changes in parasite inoculum size.

Two main observations arise from this co-infection study, the first is the reduction in the growth of the pathogenic DS by the presence of the non-pathogenic DK, and the second is the increase in the growth of DK in the presence of DS.

To address the first point, in all mixed infections with DK, DS never reached the same level of parasitaemia in a mixed infection as it did alone, even when it was the dominant strain in the infection. In contrast, growth of more pathogenic strains of the related sub-species *P. c. chabaudi* tends to be unaffected by growth in the presence of less pathogenic strains in mixed infections (DeRoode et al. 2004; DeRoode et al. 2005b; Bell et al. 2006). The reduction in the growth of the non-pathogenic DK strain by the pathogenic DS strain was an outcome more commonly found in equal

co-infections of *P. chabaudi*, but here it was only apparent with DS at a considerable excess over DK in the starting inoculum.

Evidence from super-infection studies shows that less pathogenic parasites can reduce the growth of more pathogenic parasites, however the exact timing of the inoculation of the less pathogenic parasite before the more pathogenic parasite was crucial, and depended on the particular strains involved (Snounou et al. 1989; Read and Taylor 2001; DeRoode et al. 2005a; Voza et al. 2005). For example, infection with the less pathogenic *P. chabaudi* AS-pyr1A strain 11 days prior to the more pathogenic AJ strain reduced the growth of AJ and thus protected mice from its pathogenic effects (DeRoode et al. 2005a). However this suppressive effect of AS-pyr1A was not seen when it was inoculated 3 days before or 3 or 11 days after AJ. Similarly, inoculating the *P. yoelii* 17X 1.1 strain 7 days before or up to 2 days after *P. berghei* ANKA infection, resulted in an inhibition of the cerebral malaria-like pathology attributable to *P. berghei* ANKA (Voza et al. 2005). This was apparently due to a decrease in its level of parasitaemia, but such protection was lost if the *P. yoelii* strain was given 4 or 7 days after *P. berghei* ANKA.

Given that mixed infections in natural malaria parasite populations might occur through super-infection or equal or unequal co-infection, a range of interactions between non-pathogenic and pathogenic parasites seems likely, depending on the particular combination of strains and timings. Therefore, the extent of the role of less pathogenic malaria parasite strains in modulating the growth of more pathogenic

strains in natural mixed infections must be considered, although a further analysis of this point is beyond the scope of this study.

The second key observation is that the presence of the pathogenic DS in a mixed infection actually increased the growth of the non-pathogenic DK under some starting conditions. DK reached higher levels of parasitaemia than single infection controls when it was inoculated at an equal starting ratio with DS, or at a 10 or 100-fold majority over DS. There are, however, very few examples in the literature of the enhancement of the growth of one rodent malaria parasite strain or species by another, in the first phase of infection (experiments described in (Richie 1988) and (Snounou et al. 1992)).

In several other rodent malaria studies there is some evidence of less pathogenic strains growing to a higher parasitaemia than their single infection controls, in the presence of more pathogenic strains, but this was often in super-infections or in the recrudescent phase of co-infections (DeRoode et al. 2004; Bell et al. 2006). It has been suggested that in an infection that has previously been dominated by another strain and is entering the recrudescent phase, the enhancement could be due to the immune system focussing on the majority parasite and thus allowing the minority parasite to grow up, or alternatively, due to antigenic escape of the minority parasite (DeRoode et al. 2004). However, it seems likely that an immune response would be able to target more than one antigen, or parasite strain, at a time, regardless of their relative proportions in the host. The advantageous effect of DS on DK might be

explained, for example, if DS infection led to a general suppression of the host immune response which consequently benefited the growth of DK.

Another possibility that could account for the increased growth of one parasite strain in the presence of another, is that a parasite strain with a normocyte invasion preference might lead to the greater production of reticulocytes in the host due to the ensuing anaemia, and this could benefit a co-infecting, reticulocyte-preferring parasite strain (Richie 1988). Unlike the normally reticulocyte-restricted *P. yoelii*, however, both *P. c. chabaudi* and *P. c. adami* are capable of infecting reticulocytes and normocytes in equal proportions (Carter and Walliker 1976; Jarra and Brown 1989). From Chapter 2 it is clear that in single infections, DK is approximately twice as selective as DS in its invasion of red blood cells, but this is not due to a DK preference for reticulocytes. Therefore DK invasion is apparently restricted to a sub-population of normocytes. If this is the factor that restricts the maximum parasitaemia of DK, then the presence of DS might increase DK growth if it makes available more red blood cells that are permissive for DK invasion. Again, one can only speculate as to exactly how this might occur.

Whatever the mechanisms that may lead to the increased growth of DK and the reduction of DS growth under certain conditions, the present study demonstrates how two closely-related malaria parasites that differ in pathogenicity can modify each other's growth. Evidence from other co-infection studies suggests that more pathogenic rodent malaria strains are more successful in mixed infections than less pathogenic strains (DeRoode et al. 2005b; Bell et al. 2006). Although, in super-

infection studies, less pathogenic strains introduced at the right time can reduce the growth or pathogenicity of more pathogenic strains (Snounou et al. 1989; Read and Taylor 2001; DeRoode et al. 2005a; Voza et al. 2005). However, here, using the same two strains, DS and DK, in different infection ratios, both were found to be capable of reducing each other's growth despite their different pathogenicities in single infections. An enhancement of the growth of the slow-growing DK parasite in the presence of the fast-growing DS strain was also seen.

Therefore, it may be difficult to predict how a particular malaria parasite will grow in a mixed infection because this may depend on many factors: the number and type of co-infecting strains/species involved, the proportions of these different strains relative to one another and whether the infection is a simultaneous co-infection or a super-infection. How the fluctuating levels of pathogenic and non-pathogenic parasites over time in a single host may relate to their individual infectivity to mosquitoes and the overall pathogenicity of the infection, will ultimately determine their individual success in natural infections.

In terms of the immediate relevance to the objective of this thesis, it is evident that interactions between these two particular strains do occur, and these interactions have positive and negative effects on growth depending upon the circumstances. This is important to be aware of given that mixed infections are necessary to carry out the genetic analysis and, therefore, these interactions cannot be avoided. In the light of the results from Chapters 2 and 3, some considerations can be made. Firstly, increasing the amount of DS relative to DK in a mixed infection, in order to improve

the chances of equal transmission of both strains for a cross, must be balanced against the prospect of DS suppressing the growth and perhaps, transmission, of DK. Secondly, although the slow-growing DK parentals are expected to be lost from the cross progeny pool by fast-growth selection, this might not be the case if DK growth is increased by the presence of DS parentals. Finally, as long as the cross progeny pool is not swamped by slow-growing DK parentals which might have a suppressive effect, it should be possible to select a recombinant cross progeny pool whose members have fast-growth phenotypes like the DS parent. Therefore, with these insights, the main experimental work towards a genetic analysis of the difference in growth characteristics of DS and DK can now proceed.

Chapter 4: LGS Analysis of the Difference in Growth of *P. c. adami* Strains DS and DK: Part 1.

4.1. Introduction

The large difference in growth between *P. c. adami* DS and DK, as examined in Chapter 2, is the main reason for choosing these particular rodent malaria parasites for Linkage Group Selection (LGS) analysis. Unlike the YM line of *P. yoelii* which has a very fast and lethal growth phenotype in mice and has been the subject of a recent LGS analysis (Pattaradilokrat 2008), the fast growth characteristic of DS has not arisen during use in the laboratory, but was evident in the original thicket-rat isolate from which this strain was cloned (Carter and Walliker 1976). Therefore DS and DK are examples of two naturally occurring rodent malaria parasites which are highly related (Perkins et al. 2007) and yet which differ significantly in growth and thus, pathogenicity.

As outlined in the introduction to this thesis, it is also clear that some naturally occurring isolates of the human malaria parasite *P. falciparum* can have distinct growth and invasion characteristics which may be linked to their pathogenicity in some geographical regions (Chotivanich et al. 2000; Dondorp et al. 2005). Therefore, an investigation of the genetic determinants of growth and pathogenicity in *P. c. adami* could give useful insights into the biology of other malaria parasite species. This fourth chapter comprises the results of initial LGS experiments using DS and DK, and these experiments are then extended in Chapter 5.

LGS, a genetic technique for rapid gene discovery in rodent malaria parasite infections developed in this laboratory, uses uncloned cross progeny rather than individual clones (Carter et al. 2007). It has previously been applied to examine the genetic basis for selectable phenotypes such as strain-specific protective immunity (Martinelli et al. 2005b; Pattaradilokrat et al. 2007), drug resistance (Culleton et al. 2005; Hunt et al. 2007) and most recently, growth differences in *P. yoelii* (Pattaradilokrat 2008).

Two rodent malaria parasite strains that differ in a selectable phenotype can be crossed in the mosquito vector, with the result that after meiosis the progeny will be a mixture of haploid parental genotypes and recombinants (Walliker et al. 1971; Walliker et al. 1975). These cross progeny, in the form of sporozoites from the mosquitoes in which the crossing took place, are pooled and inoculated into a rodent host. They are then subjected to a selection pressure specific to one of the parental strains and the pattern of inheritance of parental genome-wide markers (e.g. Amplified Fragment Length Polymorphism (AFLP) markers (Vos et al. 1995; Grech et al. 2002; Martinelli et al. 2004) is determined in the remaining parasite pool. Rather than using a drug or an immune response to deliver a selection pressure, for growth-phenotype LGS, fast-growing recombinants are expected to outgrow slower ones within an infection. Slow-growing recombinants, and thus markers associated with slow growth, are lost from the selected cross progeny pool. Identification of the location of these markers holds the key to discovering parts of the genome conferring the phenotype of slow or fast growth.

In this chapter, two crosses (A and B) were generated between DS and DK, and AFLP marker inheritance was analysed in cross progeny selected for fast growth. In **the** light of findings from these crosses, backcrosses were made between the slow-growing parent DK and the fast-growing cross progeny from crosses A and B. The resulting three backcrosses (1, 2 and 3) were also selected for fast growth and the same AFLP markers analysed. Marker inheritance was compared in crosses A and B, and backcrosses 1, 2 and 3. Several DK markers that were consistently reduced across these five experiments were identified, and their locations found in the *P. chabaudi* genome.

4.2. Materials and Methods

4.2.1. Parasites, Mice and Mosquitoes

As described in section 2.3.1 of this thesis, slow-growing and fast-growing strains DK and DS, of the rodent malaria parasite *P. c. adami*, were used in these experiments. DS and DK were blood-passaged a total of 5 times from cryopreserved isolates, before 1) being used to create mixed infections for the genetic crosses described in this chapter, and 2) to provide parental DK and DS DNA for AFLP analysis (see section 4.2.7 below). Six to eight week old CBA female mice were used for sporozoite-induced infections and blood stage passage, and 6-8 week old C57 female mice were used for mosquito transmissions. *Anopheles stephensi* mosquitoes used for transmission of the parasites were from a laboratory colony, maintained as detailed in section 2.3.1 of this thesis. Experimental mosquito cages contained around 100 female mosquitoes.

4.2.2. Mosquito Transmission

On the day of a mosquito feed, mice were anaesthetised with an intra-muscular injection of 50µl Rompon/Vetalar and placed on mosquito cages for 15-30 minutes, before each mouse was removed and immediately euthanased whilst still under anaesthesia. A fifteen-minute blood feed using an uninfected mouse was carried out between day 4 and day 6 post-infection in order to increase mosquito longevity. A small number of female mosquitoes were dissected on day 8 or 9 post-infection, and guts were examined for the presence of oocysts by light microscopy. If infected, all the female mosquitoes in the cage were dissected at days 17-21 of infection to remove salivary glands. Salivary glands were crushed in 50% Fetal Calf Serum 50% Ringers solution and a maximum 100µl volume per mouse was intra-peritoneally (i.p) injected.

4.2.3. Creation of Genetic Crosses and Storage of Cross Progeny

To establish a blood infection, a small volume of donor mouse blood containing the required number of parasitised red blood cells (pRBC) was mixed in either physiological citrate saline (0.15M Sodium chloride, 0.05M Sodium citrate) or 50% Fetal Calf Serum 50% Ringers solution and i.p. injected into a recipient mouse. For mixed infections, groups of three C57 mice were infected with a total of 10^6 pRBC of one of the following; a 1:1 mixture of DS:DK, a 1:5 mixture of DS:DK or a 1:20 mixture of DS:DK. On days 5, 6 and 7 of infection, one mouse per group was used for mosquito transmission. The resulting sporozoite-induced blood infections from 4 independent crosses were stored in liquid nitrogen after whole-mouse bleed-out, before infections reached 2% parasitaemia. Blood was extracted by terminal

exsanguination under anaesthesia with intramuscular 50µl Rompon/Vetalar and inhaled Halothane. Blood was collected into physiological citrate saline (approximately 1ml of blood can be expected from each mouse), spun at 3900 rpm for 5 minutes, and then the pellet was slowly reconstituted in 2 packed cell volumes (PCV) of glycerolyte. Approximately 500µl blood was frozen down in cryogenic ampoules (Corning Inc) in liquid nitrogen.

4.2.4. Estimating the Number of Recombinants in the Cross Progeny

The theoretical maximum number of recombinants present in a cross was calculated by starting with an estimate of the total number of oocysts produced by the cross:

1) Number of infected mosquitoes x average oocyst burden = total number oocysts.

Then, this value was multiplied by 4 because each oocyst will contain 4 meiotic products, thus:

2) Total number oocysts x 4 = total number of cross progeny generated.

Assuming random fertilisation among gametes of two parental lines present in equal numbers, 25% of these meiotic products should be DK parental-type, 25% should be DS parental type, and 50% should be recombinants between DS and DK. Therefore:

3) Total number of cross progeny generated ÷ 2 = total number of recombinant cross progeny.

These three equations can be simplified to:

Number recombinants in cross = (Number of infected mosquitoes x average oocyst burden) x 2

4.2.5. Retrieval of Cross Progeny from Liquid Nitrogen Storage

Progeny of the DS:DK 1:1 and 1:5 crosses from day 6 transmissions (created as outlined in section 4.2.3) were removed from liquid nitrogen storage after 22 days. Ampoules were gently thawed in water, 100µl 0.2ml 12% NaCl was added and allowed to stand at room temperature for 5 minutes. Drop-wise over at least 5 minutes, 4.5ml 1.6% NaCl was added and mixed continually. After centrifuging at 2000 rpm for 5 minutes, the pellet was resuspended in 500µl 1.6% NaCl. Dropwise over at least 5 minutes, 4.5ml 0.9% NaCl / 0.2% glucose was added and mixed continually. After centrifuging at 2000 rpm for 5 minutes, the pellet was resuspended in 0.9% NaCl / 0.2% glucose to 50% haematocrit. This suspension was directly injected i.p. at 0.1ml per mouse.

4.2.6. Growth Selection of the DS x DK *P. c. adami* Cross Progeny

After limited growth of the thawed uncloned cross progeny to a low parasitaemia (<3% cross A and <8% cross B), three rounds of growth selection, in which cross progeny were allowed to grow to high parasitaemia, were carried out. Round 1: 10^6 pRBC from cross A and B progeny were inoculated into 4 mice each and grown for 7 days. Round 2: Blood was pooled from the 3 mice with the highest parasitaemia in each group. From these pools, 10^6 pRBC from cross A and B progeny were inoculated into 4 mice each and grown for 7 days. Round 3: Blood was pooled from 4 mice in the cross A group. In the cross B group, only blood from a single mouse with the highest parasitaemia was taken. From these pools, 10^6 pRBC from cross A and B progeny were inoculated into 4 mice each and grown for 7 days (cross A) and

6 days (cross B). After this final round, blood was taken from the 2 mice with the highest parasitaemia in the cross A group and from all 4 mice in the cross B group.

4.2.7. Preparation of Parasite DNA for use in AFLP Analysis

After each round of selection, the mice used for passage in each group were bled out under terminal anaesthesia (as detailed above in section 4.2.3) and their blood was pooled by group. Blood was also taken from mice infected with either DS or DK alone. Individual sets of pooled blood were run though two cellulose and glass wool columns and chased through with sterile phosphate buffered saline (PBS). Each set of blood was further filtered through two Plasmodipur filters (Euro-Diagnostica). The filtering of the blood in this way is an effective method for removing host nucleated cells, reducing them to the level of 1 to 10 for every 10^6 parasite nucleated cells and therefore, reducing host DNA contamination (Grech et al. 2002). Blood was then centrifuged at 3000 rpm for 5 minutes, the pellet was re-suspended in PBS and centrifuged again. The pellet was then re-suspended in 2 packed-cell volumes (PCV) of 0.15% saponin (Sigma) to allow lysis of the host cells, mixed gently and an excess of PBS added to stop lysis of the released parasites themselves. After centrifugation at 4000 rpm for 5 minutes, the parasite pellet was resuspended with 5ml PBS and then centrifuged again. This was repeated until the supernatant was colourless. The saponin step was repeated only if there were un-lysed red blood cells remaining. The parasite pellet was frozen at -70°C until DNA extraction.

Parasite DNA was extracted using phenol-chloroform and then treated with RNAse A, following the method described by Grech and others (Grech et al. 2002). Briefly,

the thawed parasite pellet was re-suspended in 400ml Buffer A, 5µl 20% SDS and 2.5µl 20µg/µl Proteinase K (Roche), and incubated overnight at 37°C. 400µl of Phenol/Chloroform/IAA (Biogene Ltd) was added, mixed and centrifuged at 13,000 rpm for 60 seconds. The supernatant was taken and the previous step was repeated. 400µl chloroform was added to the supernatant and centrifuged. 40µl 3M Sodium Acetate pH 5.2 and 800µl ice-cold 100% ethanol was added to the supernatant and stored at -20°C for 60 minutes, before centrifugation at 13,000 rpm and 4°C for 30 minutes. The parasite DNA pellet was washed in 700µl 70% ethanol and centrifuged at 12,000 rpm for 120 seconds. This was repeated before allowing the pellet to dry fully. The pellet was re-suspended in 100µl pH 8 TE buffer. The parasite DNA was treated with RNAse A (Sigma) for 15 minutes at 37°C and then the concentration was measured by DNA spectrophotometry.

4.2.8. Generation and Analysis of AFLP Markers

This method is based on that described by Grech and others (Grech et al. 2002) but is also outlined below:

Step 1: Digestion of 500ng parasite DNA from DS, DK and cross progeny infections, using EcoRI and MseI restriction endonucleases (Fermentas).

Step 2: Annealing of EcoRI- and MseI-site-specific adaptors (Eurogentec). EcoRI site-specific adaptor sequences: 5'-CTCGTAGACTGCGTACC-3' and 3'-CATCTGACGCATGGTTAA-5'. MseI site specific adaptors: 5'-GACGATGAGTCCTGAG-3' and 3'-TACTCAGGACTCAT-5'.

Step 3: PCR using primers specific for the EcoRI or MseI adaptors (MWG-Biotech AG) and Taq polymerase (Promega). PCR reaction as follows: 94°C for 60 seconds

once, then 20 cycles of [94°C 30 seconds, 56°C 60 seconds, 65°C 60 seconds]. EcoRI general primer 5'-GACTGCGTACCAATTC-3' and MseI general primer 5'-GATGAGTCCTGAGTAA-3'.

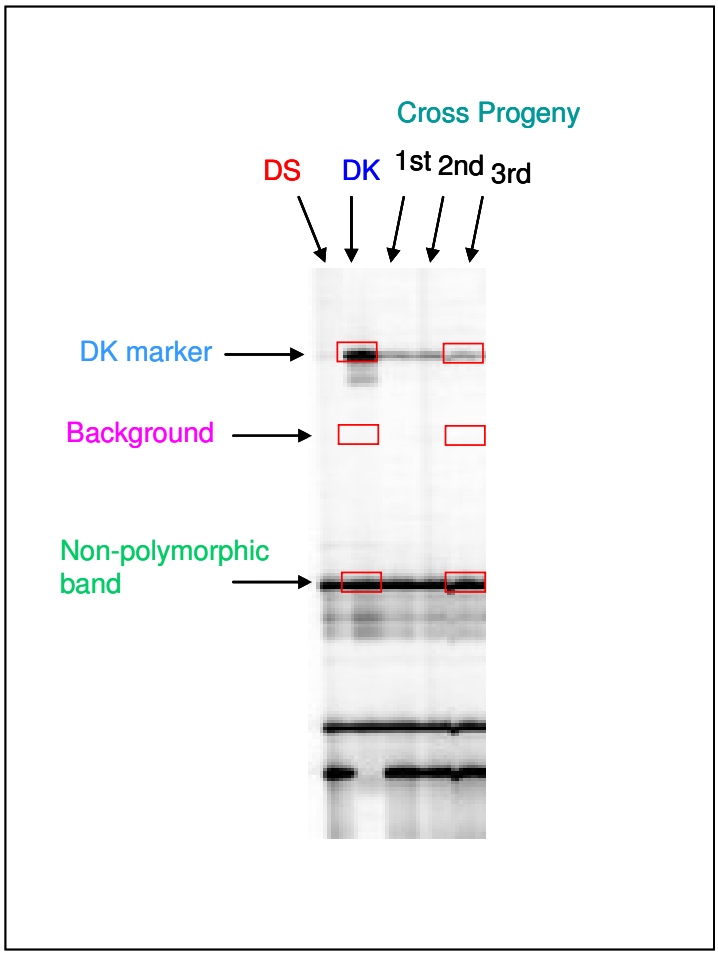
Step 4: Selective PCR using a selective MseI primer and a ^{33}P labelled EcoRI selective primer (MWG-Biotech AG). Selective primers had the same sequence as the general primers but with an additional 2 selective bases at the 3' end. PCR reaction as follows: 94°C for 60 seconds once, then 12 cycles of [94°C 30 seconds, 65°C 60 seconds reduced by 0.7°C per cycle, and 65°C 60 seconds]. This was followed by 23 cycles of [94°C 30 seconds, 56°C 60 seconds and 65°C 60 seconds].

Step 5: PCR products were loaded onto a 38cm x 50cm SequiGen 6% polyacrylamide gel (BioRad) and ran at 50°C and 110W for 1 hour. The gel was transferred onto filter paper (3MM) and dried for 2 hours under a vacuum at 80°C. It was then exposed overnight to a phosphoscreen. The screen was scanned using a phosphoimager and then analysed using ImageQuant software (version 1.2 for Macintosh by Molecular Dynamics).

4.2.9 Calculation of the Relative Intensity of a Marker in Selected Cross Progeny

Calculation of the Relative Intensities (RIs) of DK or DS specific markers found in the cross progeny following selection was achieved as follows (Figure 4.1). A band present only in the lane corresponding to the DK parent and not the DS parent, is designated a DK marker. To derive its RI in the selected cross progeny, the intensity of this band is measured in the DK parent lane of the gel, and also in the lanes corresponding to the cross progeny. As some lanes, and therefore bands, can appear

Figure 4.1
Polymorphic (marker) bands differentiating DS and DK strains were identified on a scanned image of the AFLP gel. Marker inheritance was quantified by using computer software to measure the intensity (red boxes) of the DK marker band, background and non-polymorphic band, in the DK parent and also in the cross progeny (in this example, cross progeny after the 3rd round of growth selection). Dividing the intensity of the polymorphic band (minus background) by the non-polymorphic band (minus background) gives a corrected intensity value for each cross progeny sample, and the pure parental DK sample, for the DK marker of interest. Then, the corrected intensity of the DK marker in the cross progeny sample is divided by the corrected intensity of the same DK marker in the pure parental DK sample, to give the Relative Intensity (RI) of the marker after selection.



darker than others due to background smearing, a measurement of the background is taken and subtracted from the marker band intensity in each lane. Similarly, to correct for variations in the amount of sample loaded in each lane, the intensity of a chosen non-polymorphic band on the gel is measured in the DK and cross progeny lanes. For each lane, dividing the intensity of the polymorphic band (minus background) by the non-polymorphic band (minus background) gives a corrected intensity value for each sample for the DK marker of interest. Finally, dividing the corrected intensity of the DK marker in a cross progeny sample by the corrected intensity of the same DK marker in the pure parental DK sample, gives the Relative Intensity (RI) of the marker after selection. The RI is therefore a quantification of the proportion of cross progeny that have retained that marker, relative to the parent population. The intensities of AFLP marker bands have been shown to accurately quantify the proportion of alleles in mixtures of different rodent malaria parasite strains (Martinelli et al. 2004).

4.2.10. Creation of Backcrosses

As the progeny of crosses A and B had not been stored in viable form after full selection for fast growth, it was necessary to return to the unselected cross A and B progeny that had been stored in liquid nitrogen in order to make backcrosses of the growth selected progeny to a parental line. The cross A and B progeny were, therefore, fully re-selected and then backcrossed to DK. Unselected cross A and B progeny were removed from liquid nitrogen storage, grown to <5% parasitaemia and then 10^6 pRBC were inoculated into mice to begin three rounds of growth selection. After the final round of selection, both cross progenies were used to produce mixed

infections in which selected cross progeny were mixed with DK in ratios of 1:2 and 1:4, each totalling 10^6 pRBC. These four mixed infections were grown in mice until they were transmitted to mosquitoes on day 6 post-infection. When the parasitaemias of four sporozoite-induced infections from the recombinant backcross progeny were <1%, they were pooled by backcross to give a single pool of each of the 1:2 and 1:4 backcrosses (backcrosses 1 and 2 respectively), and the two backcrosses were also mixed together give backcross 3. 10^6 pRBC of the three backcross groups were then inoculated into mice and three rounds of growth selection carried out. After each round of selection, blood was pooled and harvested for AFLP analysis.

4.2.11. Assignment of AFLP Markers to Locations in *Plasmodium* Genomes

This method is based on that described by Hunt and others (Hunt et al. 2004) . Marker bands of interest on the gel (attached by suction desiccation to filter paper) were cut out and placed into 30µl of sterile ultrapure water. The samples were centrifuged at 13,000 rpm for 10 minutes to elute the DNA and then 5µl of supernatant was used in the AFLP selective PCR reaction described above (section 4.2.8 step 4), although this time using Accuzyme (Bioline) instead of Taq polymerase. PCR products were run on a 2.5% agarose gel. If only a single, clean product band was present, the PCR product was purified with a PCR purification kit (Qiagen). If there were minor bands present, the major product band was cut out and purified with a Gel extraction kit (Qiagen). Cleaned PCR products were then sequenced using ABI Prism BigDye Terminator (Applied Biosystems) by our in-house sequencing service. If sequencing failed, the PCR products were cloned using

a TopoTA kit (Invitrogen) before plasmid extraction by Mini Prep kit (Qiagen) and then re-sequencing. Sequences were used to BLAST search the *P. chabaudi* database at the Sanger Centre (http://www.sanger.ac.uk) to identify the particular contig (un-connected sections of the *P. chabaudi* genome sequence) that contained the marker sequence. The region of the contig around the marker was then used to search the genomic databases of *P. chabaudi* and *P. falciparum* at NCBI (http://www.ncbi.nlm.nih.gov) to identify the gene in (or nearest to) this region in *P. chabaudi*, and whether it had an orthologous gene in *P. falciparum*. If an orthologue was found, use of the synteny map between *P. falciparum* and the rodent malaria parasites (Kooij et al. 2005) enabled the location of the DNA sequence to the *P. chabaudi* genome.

4.3. Results

4.3.1. Creation of Crosses Between DS and DK

Mixed infections of DS and DK were set up in order to create a genetic cross as explained in Figure 4.2a. To produce the maximum number of recombinants, it is necessary to have approximately 50% of each parent present at the time of the mosquito feed, assuming both produce equal numbers of gametocytes and that these gametocytes are equally infectious to mosquitoes. Taking into account data from Chapters 2 and 3, three different ratios of DS:DK mixed infections were set up and transmission to mosquitoes was carried out on three different days of infection. Of the 9 mice inoculated with a mixed infection, 1 mouse did not become positive for any parasites (DS:DK 1:5 day 7). Therefore only 8 were used for the mosquito

Figure 4.2
(a) Design of the transmission experiment to cross DS and DK, testing three different days of infection (5, 6 and 7) and three different starting mixtures of DS:DK (1:1, 1:5 and 1:20). Three mice in each group were infected with the particular DS:DK mixture and one mouse in each group was used to provide a blood meal to mosquitoes for transmission on a single day (marked with an X). Of the 9 mice inoculated with a mixed infection, 1 mouse did not become positive for any parasites (DS:DK 1:5 day 7) and therefore was not used for transmission. (b) The two most successful transmission events and resulting crosses A and B with their estimated numbers of recombinants.

a)

	DS:DK 1:1 mix			DS:DK 1:5 mix			DS:DK 1:20 mix		
Mouse	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Day 5:	X			X			X		
Day 6:		X			X			X	
Day 7:			X						X

b)

DS:DK mixture in starting inoculum	**Day of infection that transmission occurred**	**% Mosquitoes infected (oocysts/infected gut)**	**Number of mosquitoes dissected**	**Theoretical maximum # recombinants**
1 to 1	6	100 (32)	38	2432 CROSS A
1 to 5	6	100 (>100)	52	>10400 CROSS B

transmissions, and of these 8, only 4 produced successful transmissions. Calculation of the transmission success and the estimated number of recombinants per cross (see section 4.2.4 for method) showed that the optimum day for transmission of the mixtures was day 6 and that the 1:1 and 1:5 crosses were the most successful (Figure 4.2b). These were labelled crosses A and B respectively and were then taken forward for LGS analysis.

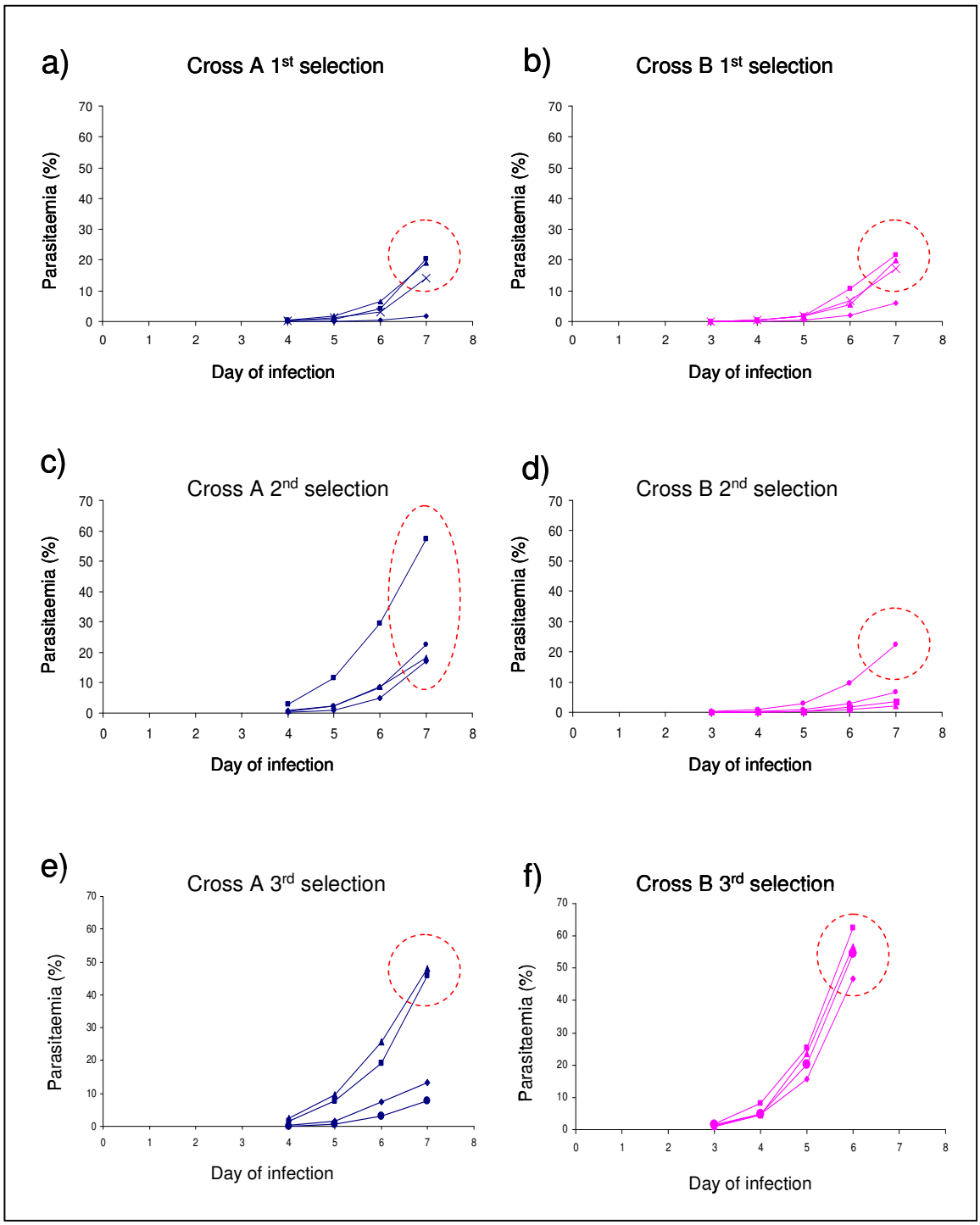
4.3.2. Growth Selection of Crosses A and B

The sporozoite-induced infections of crosses A and B were frozen down in liquid nitrogen storage on day 8 of infection when the parasitaemias were less than 2%. After being removed from storage they were grown briefly to a low parasitaemia (<3% cross A and <8% cross B) so that 10^6 parasites could be inoculated into 4 mice per cross (Figure 4.3). On day 7 when parasitaemias were sufficiently high to resemble the growth of DS and not DK (>14% parasitaemia), blood was pooled from the mice in each group with the highest parasitaemia and used to inoculate a further 4 mice per cross with 10^6 parasites. This was repeated for 3 rounds of growth selection as described in section 4.2.6. The recombinant cross progeny reached increasingly higher parasitaemias over the progressive rounds of growth selection (Figure 4.3).

4.3.3. AFLP Analysis of Cross Material

Parasite DNA extracted from pooled blood at the end of each round of selection for each cross was digested first with EcoRI and then with MseI; these are rare-cutting and frequent-cutting restriction enzymes respectively. EcoRI and MseI adaptors were then ligated to the ends of these genomic fragments and adaptor-specific primers

Figure 4.3
Selection of cross A (a, d, e) and cross B (b, d, f) progeny for fast growth over three rounds of blood passage. Each line represents the parasitaemia of an individual mouse in each group. Blue lines correspond to cross A and pink lines to cross B. 10^6 pRBCs were taken for passage at each stage, from a pool of the mice in each group with the highest parasitaemia (red dashed circle). Blood was also taken from these mice for AFLP marker analysis.



used to amplify this material by PCR. A round of selective amplification using EcoRI and MseI specific primers with 2 particular bases added to the 3' end, allowed the resolution of genomic fragments into distinct bands when run on a polyacrylamide gel. Without this selective amplification, there would be so many DNA fragments that only a smear would be seen. The EcoRI selective primers were labelled with the radioisotope ^{33}P so that once the material had been run on a gel, markers could be visualised by exposing the dried gel to a phosphoscreen. The screen was scanned by a phosphoimager to produce an image file from which markers could be quantified using computer software. The intensities of AFLP marker bands have been shown to quantify the proportion of alleles in mixtures of different rodent malaria parasite strains (Martinelli et al. 2004). Calculation of the Relative Intensities (RI) of DK or DS specific markers found in the cross progeny is described in section 4.2.9 above. Thirty-five combinations of selective EcoRI and MseI primers generated 66 DK and 60 DS AFLP markers which could be compared between crosses A and B (Figure 4.4).

4.3.4. Pattern of DK Marker Inheritance in Crosses A and B

The RI values of the 66 DK markers following 1st, 2nd and 3rd passages were compared in the progeny of crosses A and B (Figure 4.5a). There was a strong correlation between the RI of DK markers from crosses A and B following fast-growth selection, and this was maintained over successive selections: Spearman's Rank Correlations 1st passage $r_s = 0.771$ $p<0.01$, 2nd passage $r_s = 0.699$ $p<0.01$, 3rd passage $r_s = 0.731$ $p<0.01$. Around 50% of the DK markers had been reduced to a RI of less than 0.2 in both crosses, therefore around half the DK markers were inherited

Figure 4.4
35 combinations of selective EcoRI and MseI primers were used to generate 66 DK and 60 DS AFLP markers. The number of markers generated by a single selective primer combination is shown in each box. Due to the high A-T content of the *Plasmodium chabaudi* genome, selective primers that were C/G-rich were not used (n/d) since they were unlikely to generate many markers (Grech et al. 2002).

a) DK specific AFLP markers Total = 66

EcoR1 selective primer

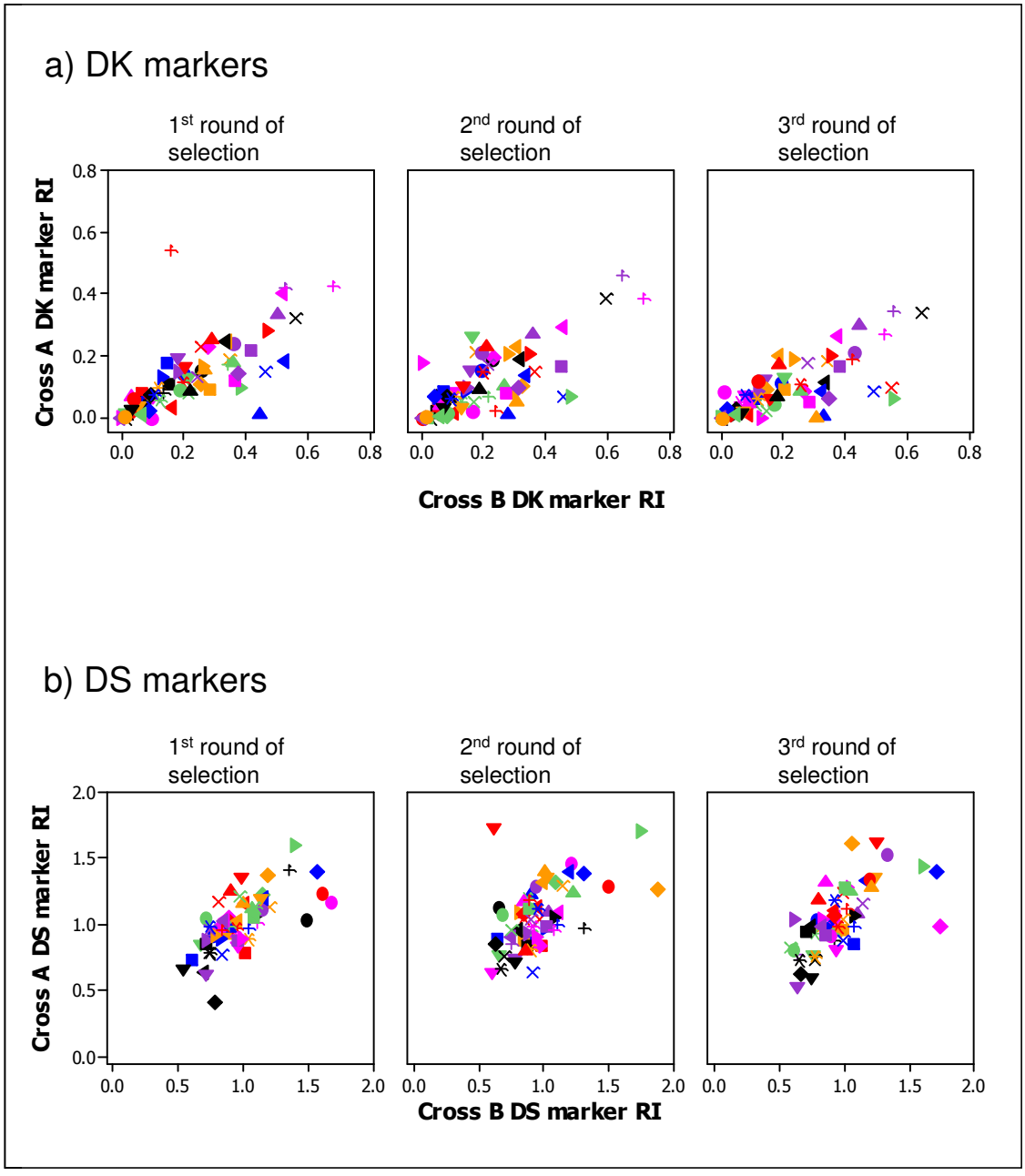
Mse1 selective primer	E-aa	E-ac	E-ag
M-aa	3	2	4
M-ac	2	1	3
M-ag	2	2	1
M-at	2	2	1
M-ca	5	1	1
M-cc	n/d	n/d	n/d
M-cg	n/d	n/d	n/d
M-ct	4	2	2
M-ga	1	1	0
M-gc	n/d	n/d	n/d
M-gg	n/d	n/d	n/d
M-gt	2	1	0
M-ta	1	n/d	0
M-tc	3	2	3
M-tg	2	3	2
M-tt	3	2	0

b) DS specific AFLP markers Total = 60

EcoR1 selective primer

Mse1 selective primer	E-aa	E-ac	E-ag
M-aa	3	2	1
M-ac	2	1	0
M-ag	1	0	2
M-at	4	4	3
M-ca	5	0	3
M-cc	n/d	n/d	n/d
M-cg	n/d	n/d	n/d
M-ct	1	0	3
M-ga	2	0	1
M-gc	n/d	n/d	n/d
M-gg	n/d	n/d	n/d
M-gt	2	0	0
M-ta	0	n/d	2
M-tc	3	1	1
M-tg	3	0	3
M-tt	4	0	3

Figure 4.5
A comparison of the DK marker (a) and DS marker (b) Relative Intensities (RI) in crosses A and B over the 1st, 2nd and 3rd rounds of fast growth selection.



by less than 20% of the cross progeny selected for fast growth.

4.3.5. Pattern of DS Marker Inheritance in Crosses A and B

The RIs of 60 DS markers following 1st, 2nd and 3rd passages were compared in the progeny of the two crosses (Figure 4.5b). Again, there was a good correlation between the RI of DS markers from crosses A and B following growth selection, and this was maintained over successive selections: Spearman's Rank Correlations 1st passage $r_s = 0.724$ $p<0.01$, 2nd passage $r_s = 0.540$ $p<0.01$, 3rd passage $r_s = 0.669$ $p<0.01$. Whereas the RIs of most DK markers were reduced, DS marker RI values clustered around 1.0. Therefore most DS markers were inherited by most of the fast-growing cross progeny. A number of DS markers had RI values >1.

4.3.6. Creation of Backcrosses Between Cross Progeny and DK

From the end of the first round of selection onwards, all DK markers had RI values between 0.0 and 0.4 in cross A, and 0.0 and 0.6 in cross B. Therefore, the DK content of both crosses was low compared to DS, and so it appeared that DS had transmitted better than DK in both these crosses. Although many DK markers had highly reduced RI values, if the level of DK in the cross progeny was low to start with, there would have been a decreased sensitivity to detect varying degrees of marker reduction. Consequently, it was necessary to introduce more DK into the genomes of the cross progeny to establish which markers were actually the most reduced, and therefore, at the very bottom of any selection valley. In order to achieve this, backcrosses were made by crossing fully-selected, fast-growing cross A and B progeny to the slow-growing parent DK.

As outlined in section 4.2.10, unselected cross progeny from crosses A and B were taken out of storage and re-selected for fast growth three times. After three rounds of selection, these cross progeny had the same fast-growth characteristics of the original selected cross progeny (data not shown). Four backcrosses were made as detailed in section 4.2.10 and illustrated in Figure 4.6. Different ratios of the cross progeny to DK were used in the starting inoculum in order to try and maximise the chances of equal proportions of gametes, from the cross progeny and DK, being taken up by the mosquitoes. Transmission success was lower than in the initial crossing experiment, therefore, in order to increase the estimated number of backcross recombinants, the backcrosses were pooled to give backcrosses 1, 2 and 3 (Figure 4.6).

4.3.7. Growth Selection of Backcrosses 1, 2 and 3

Backcrosses 1, 2 and 3 were selected for fast growth by passage in the same way as for crosses A and B (Figure 4.7). A single mouse for each backcross was used in the first round of selection, then 3 mice per backcross for the second and third rounds of selection. By days 8 or 9 of infection of the final round of selection, all three backcrosses had reached parasitaemias higher than DK, but lower than those of cross A and B progeny at the same stage of selection.

4.3.8. Pattern of DK Marker Inheritance in Backcrosses 1, 2 and 3

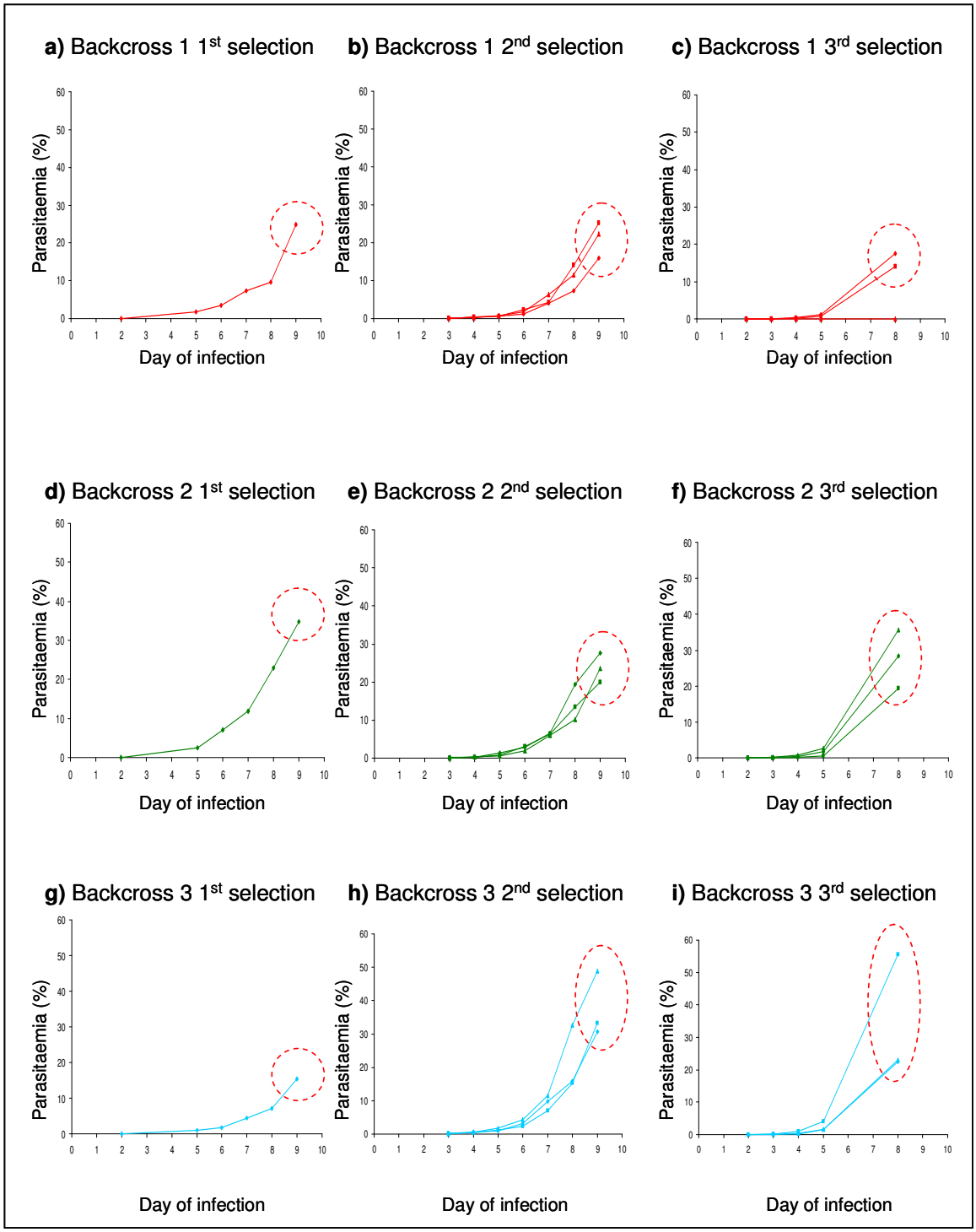
AFLP marker analysis was carried out on backcross material collected after the 1st, 2nd and 3rd rounds of passage, using the same combinations of selective primers as used for the crosses to enable a direct comparison. As expected, the proportion and intensity of DK markers increased in all 3 backcrosses. However, the pattern of

Figure 4.6
The transmission success and numbers of recombinants generated by backcrossing fully selected cross A and B progeny to DK, and the generation of backcross pools 1, 2 and 3.

Original fully selected cross used in backcross	Cross:DK in starting inoculum	% Mosquitoes infected (mean oocysts / infected gut)	Number of mosquitoes dissected	Theoretical maximum # recombinants	Pools for selection	
Cross A	1 to 2	85 (5.7)	69	669	Back-Cross 1	Back-Cross 3
Cross B	1 to 2	60 (1.2)	64	92		
Cross A	1 to 4	100 (13.6)	74	2013	Back-Cross 2	
Cross B	1 to 4	60 (2.2)	73	193		

Backcross pool	Theoretical maximum # recombinants
Backcross 1	761
Backcross 2	2206
Backcross 3	2967

Figure 4.7
Selection of backcross 1 (a-c), backcross 2 (d-f) and backcross 3 (g-i) progeny for fast growth over three rounds of blood passage. Each line represents the parasitaemia of an individual mouse in each group. Red lines correspond to backcross 1, green lines to backcross 2 and blue lines to backcross 3. 10^6 pRBCs were taken for passage at each stage, from a pool of the mice in each group with the highest parasitaemia (red dashed circle). Blood was also taken from these mice for AFLP marker analysis.



distribution of DK marker RIs in the backcrosses was strikingly different from that in the original crosses A and B (c.f. Figures 4.5 and 4.8). By comparing backcross 1 with either backcross 2 or 3, markers were distributed into four quite tight clusters. These clusters were: (1) markers that were reduced in both backcrosses, (2 and 3) those that were reduced in one backcross but not another and (4) those not reduced in either backcross. However, when backcrosses 2 and 3 were compared, marker intensities were strongly correlated, especially by the third round of selection: Spearman's Rank Correlation $r_s = 0.798$, $p<0.01$.

4.3.9. Pattern of DS Marker Inheritance in Backcrosses 1, 2 and 3

The distribution of DS marker RI values is shown as a comparison between all three backcrosses after 3 rounds of selection (Figure 4.9). In striking contrast to the inheritance of DS markers in crosses A and B (Figure 4.5), many DS markers were reduced to zero in backcrosses 1, 2 and 3. Like the DK marker distribution, DS marker RIs also fell into distinct groups, after 3 rounds of selection for fast growth, when backcross 1 is compared with either backcross 2 or 3. These clusters were: (1) markers that were reduced in both backcrosses, (2 and 3) those that were reduced in one backcross but not another and (4) those not reduced in either backcross. As with DK markers however, DS marker intensities were strongly correlated in backcrosses 2 and 3 after full selection (Figure 4.9): Spearman's Rank Correlation $r_s = 0.949$, $p<0.01$.

Figure 4.8
A comparison of the DK marker Relative Intensities (RI), over the 1st, 2nd and 3rd rounds of fast growth selection, between backcrosses 1 and 2 (a), backcrosses 1 and 3 (b) and backcrosses 2 and 3 (c).

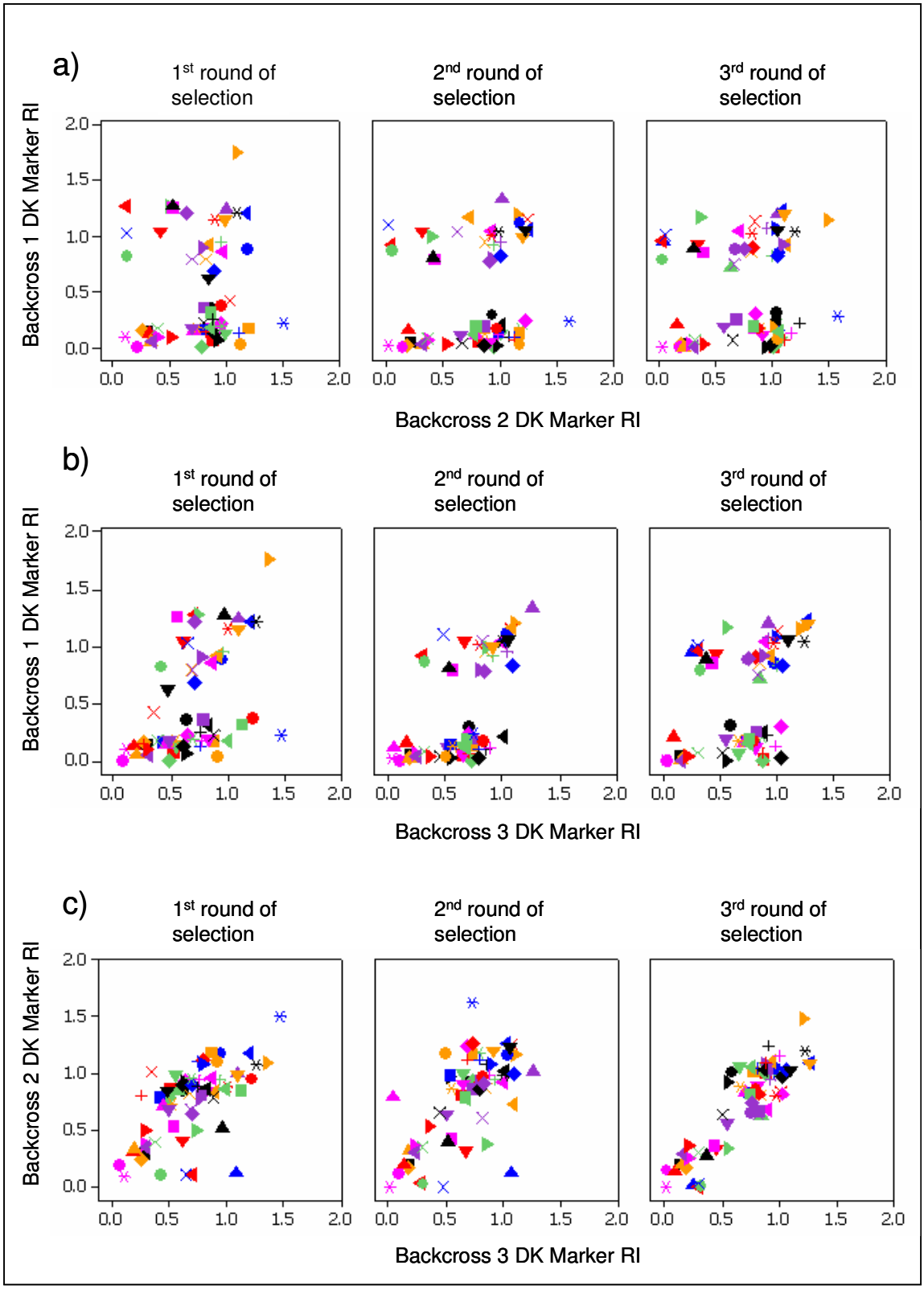
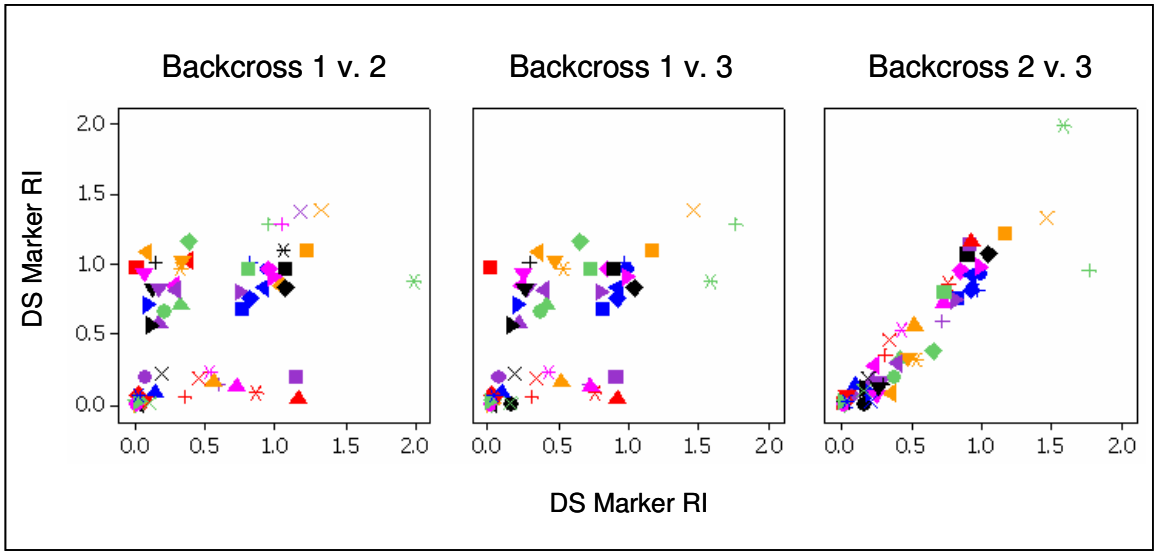


Figure 4.9
A comparison of the DS marker Relative Intensities (RI) by the 3rd round of fast growth selection, between backcrosses 1 and 2, backcrosses 1 and 3, and backcrosses 2 and 3.



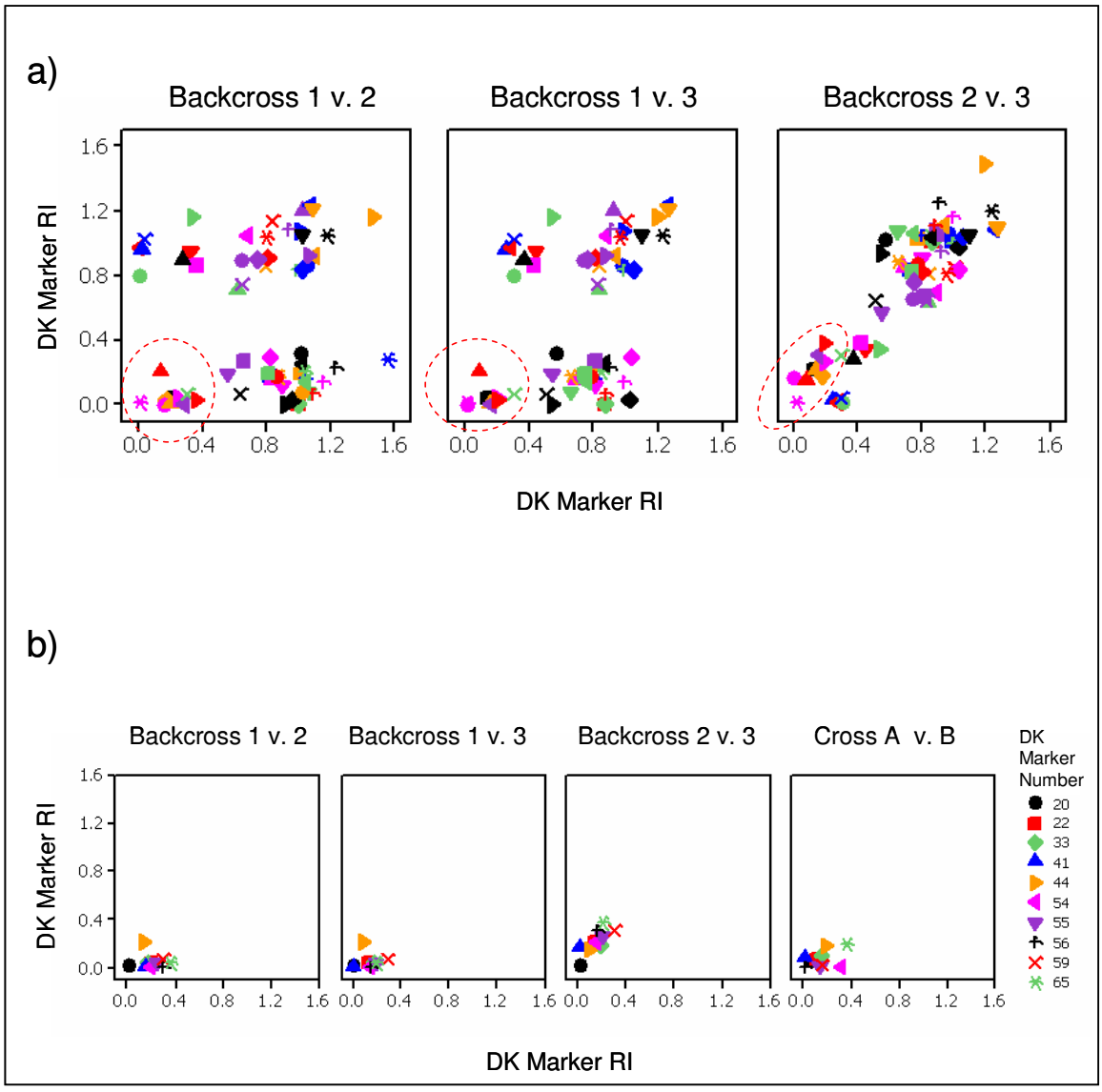
4.3.10. Comparison of DK Marker Inheritance Between Crosses and Backcrosses

A direct comparison of DK marker RI values in crosses after three rounds of growth selection show clearly the increased background level of DK and the increased range of DK marker RI values achieved by backcrossing (Figure 4.10a). However, there is a group of 10 DK markers which are consistently highly reduced by selection of all three backcrosses for fast-growth (blue circle). These were also among the highly reduced DK markers in both crosses (Figure 4.10b). This group of 10 DK markers would be expected to be closely linked to genes involved in growth determination and to fall near the bottom of any associated selection valleys.

4.3.11. Location of Reduced DK Markers in *P. chabaudi* Genome

The 10 most consistently reduced DK markers identified above (marker numbers 20, 22, 33, 41, 44, 54, 55, 56, 59, 65) were excised from their respective AFLP gels, the DNA amplified by PCR and then sequenced. If sequencing failed, it was either because the marker size was less than 100bp, or multiple PCR products of different sizes had been amplified from a single marker band. In the former case, these PCR products were cloned and re-sequenced, in the latter case the major product bands were cut directly out of gels after PCR (leaving behind minor bands) and then sequenced. These refinements yielded clear sequences for 7 of the 10 markers. The other 3 markers (54, 55 and 56) could not be assigned a definite sequence, despite repeated PCRs, gel extraction and cloning. These three markers were all on the same AFLP gel (selective primer combination EcoRI-AG, MseI-AC) and it is possible

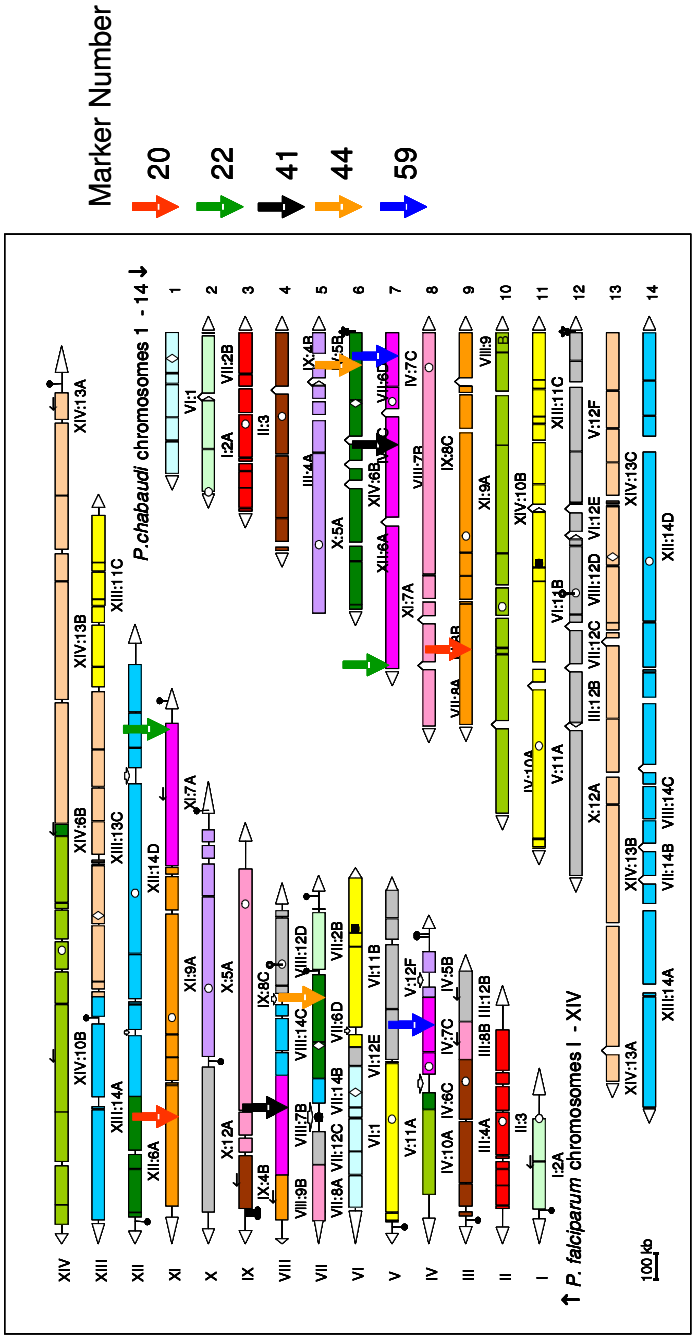
Figure 4.10
(a) A comparison of the DK marker Relative Intensities (RI) by the 3rd round of fast growth selection, between backcrosses 1 (*y-axis*) and 2 (*x-axis*), backcrosses 1 (*y-axis*) and 3 (*x-axis*), and backcrosses 2 (*y-axis*) and 3 (*x-axis*). Ten consistently most reduced DK markers are circled by a red dashed line in the three panels. (b) The RI values of only the ten consistently reduced DK markers are compared between backcrosses 1 (*y-axis*) and 2 (*x-axis*), backcrosses 1 (*y-axis*) and 3 (*x-axis*), and backcrosses 2 (*y-axis*) and 3 (*x-axis*) and crosses A (*y-axis*) and B (*x-axis*).



that this gel in particular had a high background level of DNA smearing. When the sequences of clones were examined, all were different, suggesting DNA fragments of similar size were co-migrating on the gel with these marker bands.

The locations of the remaining 7 marker sequences were sought in the *P. chabaudi* genome. Marker sequences were used to BLAST search the *P. chabaudi* genome database (based on the *P. c. chabaudi* AS strain) hosted by the Sanger Institute and 6 out of 7 sequences matched sections of the genome (contigs). One marker (number 65) did not match any sequence in the *P. chabaudi* genome sufficiently to be able to assign it to a specific contig. A further marker (number 33) was found in a gene from the phospholipase multi-gene family and therefore the exact orthologue in *P. falciparum* could not be found. In addition, none of the other *P. chabaudi* genes on the same contig as marker 33 had orthologues in the *P. falciparum* genome. The remaining 5 markers, which had good sequences and were found in or near to genes which had orthologues in *P. falciparum*, could be located using data from the *P. chabaudi – P. falciparum* synteny map (Kooij et al. 2005) (Figure 4.11). Three markers were placed on *P. chabaudi* chromosome 7, one on chromosome 6 and one on chromosome 9.

Figure 4.11
The *P. falciparum – P. chabaudi* synteny map showing the locations of 5 reduced DK markers (coloured arrows) in both parasite genomes (Figure adapted from (Kooij et al. 2005). The relative intensities of all 10 reduced markers after the third round of fast growth selection, and the *P. chabaudi* genes in which these markers fall, is shown in the table below.



DK marker number	Relative intensities of DK markers after 3rd round of selection					*P.chabaudi* gene in which DK marker falls	location of orthologous gene in *P.falciparum*	Location in *P.chabaudi* genome using synteny
	CROSS A	CROSS B	BACKX 1	BACKX 2	BACKX 3			
20	0.05	0.05	0.01	0.01	0.01	PC000196.05.0	chromosome 11 : PF11_0153	**chromosome 9**
22	0.06	0.10	0.04	0.21	0.13	near to PC2000028.00.0 (*cir* gene family)	no orthologue but end of *P.chabaudi* contig matches end of chromosome 11	**chromosome 7**
33	0.09	0.14	0.03	0.18	0.18	PC000708.00.0 (*phospholipase* gene family)	no orthologue and only on short contig	?
41	0.08	0.00	0.00	0.17	0.00	near to PC000571.02.0	chromosome 8 : MAL8P1.79	**chromosome 7**
44	0.17	0.18	0.21	0.15	0.07	PC001084.02.0	chromosome 7 : PF07_0086	**chromosome 6**
54	0.00	0.30	0.00	0.21	0.14	cannot sequence		?
55	0.00	0.12	0.03	0.26	0.19	cannot sequence		?
56	0.00	0.00	0.00	0.30	0.15	cannot sequence		?
59	0.02	0.15	0.06	0.31	0.29	PC000503.01.0	chromosome 4 : PFD0855c	**chromosome 7**
65	0.20	0.35	0.03	0.37	0.19	not in *P.chabaudi* AS genome		?

4.4. Discussion

Two independent genetic crosses between *P. c. adami* DS and DK have been studied by selecting for fast growth over three rounds of passage in mice. These two crosses were chosen from a total of four because they had the highest numbers of expected recombinants based on transmission success (numbers of mosquitoes infected and oocyst densities per mosquito). These crosses were made by transmission of DS:DK mixtures to mosquitoes on day 6 of the blood infection. Of the three days tested, day 6 of infection seemed to be the optimum day for transmission, and this is in close agreement with the transmission profiles of the two strains individually as demonstrated in Chapter 2.

After the first round of growth in mice, the cross progeny pools from both cross A and B reached parasitaemias of around 20%. By passaging on from mice with only the highest parasitaemias each time, this resulted in cross progeny reaching 50-60% parasitaemia by day 6 or 7. This is, in fact, a faster growth rate than expected from the same 10^6 pRBC inoculum of the pure DS parental strain (as shown in Chapter 2). It is possible that by crossing the two strains, recombinants have been generated that are faster growing than either parent because potentially, elements with negative and positive effects on growth could exist in the genomes of both parents. Selection would therefore have favoured those recombinants inheriting the most positive elements. Another explanation could be the result of some combination of interactions between DS and DK, as examined in Chapter 3. Finally, the passage of DS or DK alone for three rounds, in the same way as the cross progeny, could also

result in increased growth, however, during the use of DS for this thesis, such an increase in growth by sequential passage has not been noted.

The cross progeny pools collected after each round of selection were analysed for their inheritance of DS and DK-specific AFLP markers. Interestingly, from a comparison of the same EcoRI and MseI selective primer combinations used to analyse *P. c. chabaudi* strains AS and AJ (Grech et al. 2002), fewer markers were found to differentiate DS and DK, than were found to differentiate AS and AJ. For the combinations giving 59 DK markers and 53 DS markers in the present study (out of a total of 66 DK markers and 60 DS markers respectively), 73 AS markers and 72 AJ markers were found. This may suggest that DS and DK are more closely related than AS and AJ.

Comparison of DK marker inheritance after selection of cross progeny from crosses A and B showed that while there was a range of marker intensities overall, the levels of DK were low. A large number of DK markers were apparently absent from the cross progeny, or were inherited at low levels. Assuming a genome-wide marker distribution, this suggests that loci involved in the control of growth are spread throughout the genome. If, by contrast, the control of growth was a single locus trait, we would expect only a small proportion of DK markers to be reduced. Thus in an LGS analysis using *P. chabaudi* strains AS and AJ to investigate pyrimethamine drug resistance, which is known to be determined at a single gene locus, only 6 markers out of the 206 AJ sensitive parent markers analysed were reduced and these formed a single selection valley in the vicinity of the resistance gene (*dhfr*) (Culleton

et al. 2005). Growth determination could involve a number of different regions of the genome if there were several members of a gene family or several genes in a particular functional pathway involved.

The capacity of the AFLP technique to accurately determine the proportion of parasites in a mixture starts to decline when the proportion of either parasite drops below 10% (Martinelli et al. 2004). Therefore, the relatively low level of DK present in crosses A and B is the limiting factor in the analysis of these cross progeny by proportional AFLP, because so many DK markers have RIs of around 10% or less. The less DK present in the crosses to start with, the less significant the decrease of selected markers is, relative to non-selected markers, and therefore it is difficult to establish which markers are the most reduced.

The inheritance of DS markers by cross A and cross B progeny showed relative intensity ranges from 0.5 to >1.5. Therefore no DS markers were strongly reduced by selection for fast growth, and many markers had an RI around 1, indicating neutral inheritance. As marker bands become particularly dark if they are present in the majority of the cross progeny, due to saturation of the image on the phosphoscreen it becomes difficult to determine an accurate RI value, therefore there is a large variation in RI values of DS markers above the level of RI = 1. This means that DS markers which may appear to be increased in inheritance after selection may actually display only neutral inheritance. Therefore, finding DK markers which are reduced by selection for fast growth, rather than DS markers which are increased, is a more reliable strategy.

Both the DK and DS marker inheritance patterns were strongly correlated between crosses A and B, indicating that the two independent cross progeny populations behaved the same way after growth selection. Therefore, AFLP marker inheritance patterns appeared to be robust and reproducible.

Due to the problem of low DK representation in the crosses, either as a result of an overgrowth of DS in the mixed infection (Chapter 3), or a lack of DK transmission despite its relatively higher infectiousness compared to DS (Chapter 2), crosses A and B were backcrossed to DK. Cross progeny were re-selected for fast growth and then mixed with parental DK and transmitted. Mosquito transmission efficiencies were lower in these backcrosses, therefore pools were made to increase the number of backcross progeny, and thus, to increase the resolution of LGS analysis. Backcrosses 1, 2 and 3 were selected for fast growth, although they grew slower than the cross progeny from crosses A and B. This could have been due to the presence of a significant amount of uncrossed, DK parental parasites in the cross progeny. It is clear from Chapter 3 that DK can negatively affect the growth of DS when present in the majority in a mixed infection, therefore DK might also have an inhibitory effect on DS x DK recombinants with DS growth characteristics. Despite this however, all backcross progeny pools reached higher parasitaemias than DK typically reaches by day 8 or 9 of infection.

The inheritance of the same 66 DK markers was examined in the backcross 1, 2 and 3 progeny over selection. It was immediately clear that backcrossing increased the

overall level of DK in the cross progeny because markers had RI values ranging from zero to around 1. Backcrosses 2 and 3 correlated well after 3 rounds of selection, with a group of markers reduced below RI values of 0.5 in both backcrosses. However, DK marker inheritance by backcross 1 did not correlate with either backcross 2 or 3, as there were many markers which were reduced in backcross 1 but not reduced in backcrosses 2 and 3. Likewise, there were 8 markers which were not reduced in backcross 1 but were reduced in backcrosses 2 and 3.

In a similar way, the DS marker inheritance patterns are very complex, with some DS markers actually being selected against. Fully selected, fast growing recombinants (and DS parents) from crosses A and B must have all possessed the DS growth loci. These parasites then recombined with the slow-growing DK parental background during the backcross. DS markers not associated with fast growth which recombined onto a slow growing parasite would have been lost from the progeny pool after selection and this might explain the reduced DS marker group in the backcrosses.

The irreproducibility of certain markers' inheritance is most likely the result of the lower number of recombinants expected to be present in backcross 1 relative to backcrosses 2 and 3. Fewer recombinants decrease the resolution of the LGS technique, which requires large numbers of pooled cross progeny. In a pool of uncloned cross and backcross progeny with large numbers of recombinants, almost every frequent recombination event can be expected. Therefore individual large pools of recombinants should be genetically similar before selection for fast growth.

If the genetic composition of the cross progeny determines the growth phenotype, then all crosses should have similar marker inheritance after growth selection. However, if there are significantly smaller numbers of recombinants to start with, and thus fewer recombination events represented, marker inheritance is likely to be different after selection.

Nevertheless, there were 10 DK markers which were consistently reduced in both crosses A and B, and backcrosses 1, 2 and 3. Three of these markers could not be located for technical reasons relating to sequencing problems, two further markers (65 and 33) were sequenced but not located. The sequence for marker 65 did not match the *P. chabaudi* AS genomic database, therefore this marker is likely to be in a region of a gene, or an intragenic region, that is highly polymorphic in *P. c. adami* DK relative to *P. c. chabaudi* AS. Further sequencing out in both directions from this marker sequence would be required to find a region that was non-polymorphic, and so enable matching to the genome sequence. Therefore the current location of this marker is unknown.

Marker 33 was found to be in a gene that is a member of the phospholipase gene family. As this marker was found on a short contig of only 66kb which contained genes that were also part of gene families and/or were *P. chabaudi* specific, no gene orthologues could be found accurately in *P. falciparum*. Therefore, until this contig is joined up with others containing genes which have orthologues in *P. falciparum*, it is not possible to locate this marker. Gap closure on the *P. chabaudi* genome is on-going at the Sanger Institute and it is possible that in the future this marker will be

able to be located. Given that marker 33 is in a region containing only *P. chabaudi*-specific genes, it is likely to be located at the end of a chromosome. This is because the synteny that exists between *Plasmodium* species in the central regions of chromosomes generally breaks down in subtelomeric regions (Carlton et al. 2002; Hall et al. 2005).

The remaining 5 markers which could be sequenced and located onto the *P. chabaudi* genome reveal 3 different chromosomes (6, 7 and 9) which contain loci involved in the control of growth. Three reduced markers span the whole of chromosome 7 which is around 1.2Mb in length. Therefore, either a very large and shallow selection valley exists on this chromosome, or there is more than one selection valley. The ends of chromosomes 6 and 9 appear to contain another two loci, confirming the indications from DK marker inheritance distributions that growth is not under the control of a single genetic locus.

The strong involvement of loci on chromosome 7 and 9 is demonstrated by the fact that the two most reduced of the 10 DK markers were markers 20 and 41 (left-hand side of chromosome 9 and middle-right of chromosome 7 respectively). Therefore, a more detailed analysis of marker inheritance across chromosome 7 and 9 is required to identify the precise regions linked to the control of growth. These experiments were conducted and are described in the following chapter, Chapter 5.

Chapter 5: LGS Analysis of the Difference in Growth of *P. c. adami* Strains DS and DK: Part 2.

5.1. Introduction

The work detailed in Chapter 4 led to the identification of three *P. chabaudi* chromosomes, 6, 7 and 9, in which loci for the control of growth had been identified by LGS analysis. As the two most reduced DK markers were located on chromosomes 7 and 9, these candidate chromosomes were taken forward for further analysis. Due to time restrictions, these were prioritised over the further exploration of loci on chromosome 6.

The benefit of using AFLP markers for the LGS analysis of Chapter 4 was that no prior knowledge of the fully assembled *P. chabaudi* genome was required (Grech et al. 2002; Martinelli et al. 2004; Martinelli et al. 2005a). Markers were generated simply by digesting the genomic DNA into fragments and selectively amplifying this pool to achieve a number of markers unique to each parent. These markers might have been located using a linkage map (Martinelli et al. 2005a) had the *P. chabaudi* genome sequence and *P. falciparum* synteny map not been available. However, as the sequence and synteny information was available from the *P. chabaudi* and *P. falciparum* genomes, it was now possible to generate markers at known locations in the *P. chabaudi* genome.

Pyrosequencing™ technology (Biotage), herein referred to simply as pyrosequencing, has been adopted in this laboratory for the accurate quantification of

the presence of single nucleotide polymorphisms (SNPs) which are specific for different *P. chabaudi* strains (Cheesman et al. 2007). As explained in the introduction to this thesis, pyrosequencing can quantify the proportion of DNA in a mixture that has one of two possible nucleotides at a single position (SNP) in a small genomic region. In the case of LGS, it can, therefore, be used to determine the proportion of parasites in a cross which have inherited the SNP corresponding to one or the other of the two *P. chabaudi* parent strains (Cheesman et al 2008, manuscript in preparation). Consequently, pyrosequencing was used to measure the inheritance of DS and DK-specific SNPs on chromosomes 7 and 9, and later, on chromosome 1.

Four steps were involved in employing pyrosequencing for the experiments in this chapter. (1) A specific region of around 500bp (corresponding to part of a gene in a location of interest) was amplified by PCR and sequenced in each of the two parent *P. chabaudi* strains DS and DK. (2) By aligning the sequences, SNPs were identified between DS and DK. (3) This sequence information was used by the pyrosequencing marker design software to design forward and reverse primer sequences around a particular SNP (the marker) that it had calculated as the most favourable for allele quantification. (4) This region was then amplified in DS x DK cross progeny DNA pools and the proportion of the DNA with the DK-specific SNP at the marker position, relative to the DS-specific SNP, was measured by the instrument. This gives an accurate quantification of the proportion of cross progeny that have inherited the DS or DK marker.

Locations for pyrosequencing markers at approximately 150kb intervals across all 14 chromosomes of *P. chabaudi* AS, AJ and CB have been determined in this laboratory as part of an ongoing LGS investigation into the targets of strain-specific protective immunity (Cheesman et al 2008, manuscript in preparation). Therefore, primers based on the *P. chabaudi* AS genome sequence were already available for sequencing at several locations on chromosomes 1, 7 and 9. Consequently, DS and DK markers were generated in these locations. An additional two marker locations were required on chromosome 9 to increase coverage, and for these locations, primers were designed *de novo*.

In this chapter, DK marker inheritance on candidate chromosomes, 7 and 9, and on a neutral chromosome, chromosome 1, was analysed by pyrosequencing. Initially, crosses A and B and backcrosses 1, 2 and 3 (as described in Chapter 4) were examined. Subsequently, new DS x DK crosses C, D and E were also generated and analysed, and all crosses and backcrosses compared with each other. DK marker inheritance patterns across chromosome 7 revealed a complex situation with no consistently strong selection against any particular region. On chromosome 9, however, DK markers in a region on the left-hand side of the chromosome were strongly and consistently selected against in all fast-growing cross progeny pools. These results are discussed with regard to the location and nature of potential candidate genes for the determination of growth characteristics in *P. c. adami*.

5.2. Materials and Methods

5.2.1. Pyrosequencing Marker Design

As described above, for most marker locations a set of SNP-finding primers based on the *P. chabaudi* AS genome had been designed to amplify up a region approximately 500bp in length and were kindly provided by Dr Sandra Cheesman. In cases where markers were designed *de novo*, a *P. chabaudi* gene in the desired location was chosen using the *P. chabaudi* - *P. falciparum* synteny map (Kooij et al. 2005), and Primer 3 software (http://primer3.sourceforge.net/) was used to design appropriate forward and reverse SNP-finding primers from the gene sequence.

In both cases, these SNP-finding primers were used to amplify the particular region of interest from DS and DK genomic DNA (prepared as described in Chapter 4, section 4.2.7) by PCR. The SNP-finding PCR reaction contained 1µl DNA added to the following: 1x Immobuffer (Bioline), 1.5mM $MgCl_2$, 0.3mM dNTPs, 1 unit Immolase DNA polymerase (Bioline) and 0.3µM Forward and Reverse SNP-finding primers (Eurogentec) in a total volume of 50µl in sterile MilliQ water. The SNP-finding PCR reaction conditions were as follows: 7 minute 95˚C initial activation step, 28 cycles of [95˚C 60 seconds, 50˚C 60 seconds, 72˚C 60 seconds] and ending with a 10 minute 72˚C hold. PCR products were visualised under UV light after running on a 1.5% agarose gel stained with Safeview (NBS Biologicals) or Ethidium bromide. Products were cleaned up with a PCR purification kit (Qiagen) and then sequenced using ABI Prism BigDye Terminator (Applied Biosystems) by our in-house sequencing service. There was only 1 occasion when the SNP-finding primers based on the *P. chabaudi* AS genome sequence did not amplify *P. c. adami* DS or DK DNA and, in this case, a replacement marker was designed *de novo* at a nearby location. DS and DK sequences were aligned using ClustalW2 software

(http://www.ebi.ac.uk/Tools/clustalw2/index.html) to reveal SNPs. Pyrosequencing Assay Design Software, Version 1.0.6 (Biotage), was used to design the best assay from each set of SNPs. This generated the sequences of the pre-pyrosequencing forward and reverse primers, and the sequencing primer, which were then made by Eurogentec.

5.2.2. Extraction of Parasite DNA for Pyrosequencing Analysis

DNA from crosses A and B, and backcrosses 1, 2 and 3 was prepared from the whole-mouse bleed-outs described in Chapter 4, section 4.2.7, and then stored at -20°C. From these stocks, DNA was taken for pyrosequencing analysis. For new crosses C, D and E (see below), 20µl mouse tail blood was taken and mixed with 1 ml physiological citrate saline. Samples were pelleted by centrifugation at 10,000 rpm for 60 seconds and the supernatant was removed. Blood pellets were then stored at -70°C until required, when they were thawed and resuspended in 200µl PBS. DNA was then extracted by High Pure PCR template preparation kit (Roche) according to manufacturers' instructions and stored at -20°C until required. For crosses C, D and E, DNA was also extracted from mashed infected mosquito mid-guts dried on FTA Classic Card (Whatman). A single disc per sample was cut from the card with a 1.2mm diameter micropunch (Harris). Discs were washed once in 100µl FTA reagent (Whatman) before being washed with sterile MilliQ water for 5 minutes.

5.2.3. Pre-Pyrosequencing PCR Reaction

This method is based on that of Cheesman and others (Cheesman et al. 2007) and has also been described in Chapter 3, section 3.3.3. For each PCR reaction, 1µl DNA

(between approx. 10 and 15ng) or a single washed sample FTA disc, was added to the following: 1x Immobuffer (Bioline), 1.5mM $MgCl_2$, 0.3mM dNTPs, 1 unit Immolase DNA polymerase (Bioline) and 0.3µM Forward and Reverse pyrosequencing primers (Eurogentec) in a total volume of 50µl in sterile MilliQ water. Pre-pyrosequencing PCR reaction conditions: 7 minute 95˚C initial activation step, 38 cycles of [95˚C 60 seconds, 50-56˚C (depending on the primers) 60 seconds, 72˚C 60 seconds] and ending with a 10 minute 72˚C hold. PCR products were visualised under UV light after running on a 1.5% agarose gel stained with Safeview (NBS Biologicals) or Ethidium bromide. Reactions giving a single product band of the expected size were used for pyrosequencing.

5.2.4. Pyrosequencing Reaction

This method is based on that of Cheesman and others (Cheesman et al. 2007) and has also been described in Chapter 3, section 3.3.4. In each well of a 96 well plate, 35µl Streptavidin-Sepharose high performance beads (Amersham Biosciences) in Binding Buffer solution (Biotage) were mixed with 40µl sterile distilled water and 2-10µl of the biotinylated PCR product (depending on the product yield). The PCR plate was gently shaken for 10 minutes at room temperature. The pyrosequencing Vacuum Prep Tool (Biotage) was used to take up the beads (and attached PCR product) from each well and wash them for 5 seconds each with 70% ethanol, followed by 0.2M Sodium Hydroxide, and finally wash buffer (Biotage). Using the vacuum pump, the beads and attached (now single-stranded) DNA template were then transferred into a 96-well pyrosequencing plate (Biotage) containing 0.5µM sequencing primer (Eurogentec) in annealing buffer (Biotage). This plate was heated at 80˚C for 120

seconds to anneal the sequencing primer to the DNA. Once cooled to room temperature, the plate was analysed by the PSQ HS 96A Pyrosequencer instrument. The instrument dispenses an enzyme and substrate mix into each well and then individual nucleotides in turn in a defined sequence.

Samples were run in duplicate at least, most often having several more replicates than this. Results were also often repeated with the products of at least one replicate PCR reaction. Negative controls (pre-pyrosequencing PCR reaction with no DNA template) were run with every pyrosequencing reaction. Only those allele quantifications passing automatic and manual quality control checks were included for analysis. The mean and standard error of the mean (sem) were calculated for these replicates.

5.2.5. Creation of DS-DK Control Mixtures for Pyrosequencing Marker Testing

Controlled mixtures of DS and DK infected blood were made by mixing volumes of blood containing calculated numbers of parasitised RBCs in the following DS:DK ratios in physiological citrate saline; 100:0, 90:10, 75:25, 50:50, 25:75, 10:90 and 100:0. Blood was then pelleted by centrifugation at 10,000 rpm for 60 seconds and DNA was extracted using the High Pure PCR template preparation kit (Roche) as above (section 5.2.2). These controlled mixture samples were then used to test the quantification accuracy of each individual pyrosequencing assay (Appendix 1). Data were not normally distributed; therefore correlations of the expected DK proportions (from the controlled mixtures) with their matching observed DK proportions

(measured by pyrosequencing) were calculated by Spearman's Rank correlation (Minitab, version 14).

5.2.6. Creation of Cross C and Selection for Fast Growth

The new crosses described in this chapter were made and selected in the same way as those in Chapter 4. Specifically, cross C was made from two CBA mice infected with a 1:5 ratio of DS:DK which provided a blood-meal on day 6 of infection to around 250 female mosquitoes in the same cage. On day 9 of the mosquito infection, 7 infected mosquito guts with oocyst burdens ranging from 2 to 112 were mashed and absorbed onto FTA Classic Card (Whatman) ready for DNA extraction as described in section 5.2.2 above. Due to the large number of mosquitoes subsequently dissected on day 17 of the mosquito infection, the pooled sporozoites were inoculated into two separate mice.

After 4 days of the sporozoite-induced infection when the parasitaemias of both mice were less than or equal to 1%, blood was pooled and 10^6 pRBCs passaged on into one mouse for the 1st round of fast growth selection. After 14 days, 10^6 pRBCs were passaged on into one mouse for the 2nd round of selection. After 9 days, 10^6 pRBCs were passaged on into one mouse for the 3rd round of fast growth selection, and the final blood sample was harvested on day 8. Only one mouse was required for each round of passage since unlike the AFLP technique, large quantities of parasite DNA, and therefore blood, are not required for pyrosequencing marker quantification. 20µl blood samples were taken for analysis and DNA was extracted as detailed above.

5.2.7. Creation of Crosses D and E and Selection for Fast Growth

Crosses D and E were made around six months later than cross C. Two mice infected with a 1:1 ratio of DS:DK gave rise to cross D. A further two mice infected with a 1:2 ratio DS:DK gave rise to cross E. Both mice in each group were fed on the same cage of around 250 female mosquitoes on day 6 of infection. On day 8 of the mosquito infection, 12 infected mosquito guts from cross D (with oocyst burdens ranging from 1 to >100) and 11 infected mosquito guts from cross E (with oocyst burdens ranging from 3 to 68) were mashed and absorbed onto FTA Classic Card (Whatman) ready for DNA extraction as described in section 5.2.2 above.

Sporozoite-induced infections were generated in a single mouse per cross. 10^6 pRBCs were passaged on into one mouse per cross for the 1st round of fast growth selection on day 4 at 1.93% parasitaemia for cross D and on day 3 at 0.58% parasitaemia for cross E. For the 2nd round of selection, 10^6 pRBCs of cross D and E progeny were passaged on into a single mouse each on day 13. They were then frozen down as stabilates in liquid nitrogen on day 8 of infection. For the 3rd round of selection, cross D and E stabilates were removed from storage and inoculated into one mouse per cross and grown for 6 and 7 days respectively, before again being stored in liquid nitrogen. Finally, these crosses underwent a 4th round of selection; cross D and E stabilates were removed from storage and inoculated into one mouse per cross and grown for 5 and 6 days respectively, before final blood samples were taken.

5.2.8. Estimate of the Number of Recombinants in Crosses C, D and E using Oocyst DNA Quantification

Oocyst DNA was extracted from crosses C, D and E and the average proportion of DK in the oocysts was calculated from the quantification of pyrosequencing marker inheritance. Two markers on chromosome 9 were measured for cross C, and later on, one marker on each of chromosomes 1, 7 and 9 was measured for crosses D and E. These gave averages of:

65.7% DK / 34.3% DS in cross C

(from a mixed infection initiated with 83% DK / 17% DS),

81.6% DK / 18.4% DS in cross D

(from a mixed infection initiated with 50% DK / 50% DS),

and 96.7% DK / 3.3% DS in cross E

(from a mixed infection initiated with 67% DK / 33% DS).

In the previous estimation of the number of recombinants in crosses A and B and backcrosses 1, 2 and 3 (Chapter 4, section 4.2.4), it was assumed that 50% of the oocysts would contain DK, giving rise to the estimate that 50% of the meiotic products would be recombinants. However, with the use of the pyrosequencing technique to quantify the total oocyst proportion of DK in the new crosses C, D and E, this now could give a more accurate estimation of the number of recombinants.

The following equation represents a cross between DK (a) and DS (b) according to the expected Mendelian 1:2:1 ratio of meiotic product distribution:

$$\mathbf{a^2 + b^2 + 2(ab) = 1}$$

where $\mathbf{a^2}$ is the number of DK parentals (DK x DK), $\mathbf{b^2}$ is the number of DS parentals (DS x DS) and **2(ab)** is the number of recombinants 2(DS x DK) in the cross. If it is a perfect cross and the content of total oocysts is 50% DK and 50% DS, then inputting these values gives: $(0.5)^2 + (0.5)^2 + 2(0.5x0.5)$ and therefore, the meiotic product proportions of 0.25 DK, 0.25 DS and 0.50 DS x DK recombinants. However, for crosses C, D and E, the real proportions of DS and DK could be inputted into the above equation and a more accurate number of recombinants calculated (see Table 5.1).

5.3. Results

5.3.1. Pyrosequencing (SNP) Markers on Chromosomes 1, 7 and 9

Markers across chromosomes 1, 7 and 9 were designed and tested on controlled mixtures of different ratios of DS:DK (see section 5.2.5 and Appendix 1). The proportion of DK measured by pyrosequencing was highly significantly correlated with the proportion of DK expected from controlled mixtures, indicating that pyrosequencing could accurately measure the proportions of DK in a mixture for all these marker assays (Appendix 1). The locations of the reduced DK AFLP markers on chromosome 7 identified in Chapter 4 were: marker 22 at 20kb along the chromosome, marker 41 at 867kb along the chromosome and marker 59 at 1063kb along the chromosome. For chromosome 9, the single reduced DK AFLP marker was marker 20, located 407kb along the chromosome.

To generate a detailed picture of the selection over the entirety of the two chromosomes of interest, a total of 9 markers were generated on chromosome 7 (an average of 1 every 133kb) and 11 markers on chromosome 9 (an average of 1 every 127kb). In order to detect a selection valley if it exists, it is estimated that one marker every 150kb is necessary (Carter et al. 2007). Therefore, this level of coverage across chromosomes 7 and 9 should be sufficient. In order to estimate the background level, or unselected amount, of DK present in the cross progeny pools, markers were also generated on chromosome 1. Chromosome 1 was chosen because it had not been identified as a region under selection by the AFLP marker screen in Chapter 4 and therefore it was assumed that DK markers on chromosome 1 should be inherited in a neutral way. All pyrosequencing data are presented as the % DK at a particular location along each chromosome, i.e. the percentage of the cross progeny population that have the DK marker (rather than the DS marker) at that particular SNP position.

5.3.2. Effects on Chromosome 1, 7 and 9 DK Markers After Full Growth Selection of Crosses A and B and Backcrosses 1, 2 and 3

The effects of growth selection on chromosome 1, 7 and 9 DK markers were tested on the fully fast-growth selected (3rd round) cross progeny material from crosses A and B, and backcrosses 1, 2 and 3. In this selected progeny, chromosome 7 DK marker inheritance was around 5-10% in cross A and backcross 1 progeny (Figure 5.1b) compared to 10-30% on the neutral chromosome 1 (Figure 5.1a). Chromosome 7 DK marker inheritance was around 20-30% in cross B, backcross 2 and backcross 3 progeny (Figure 5.1b). This was only a slight reduction relative to the chromosome

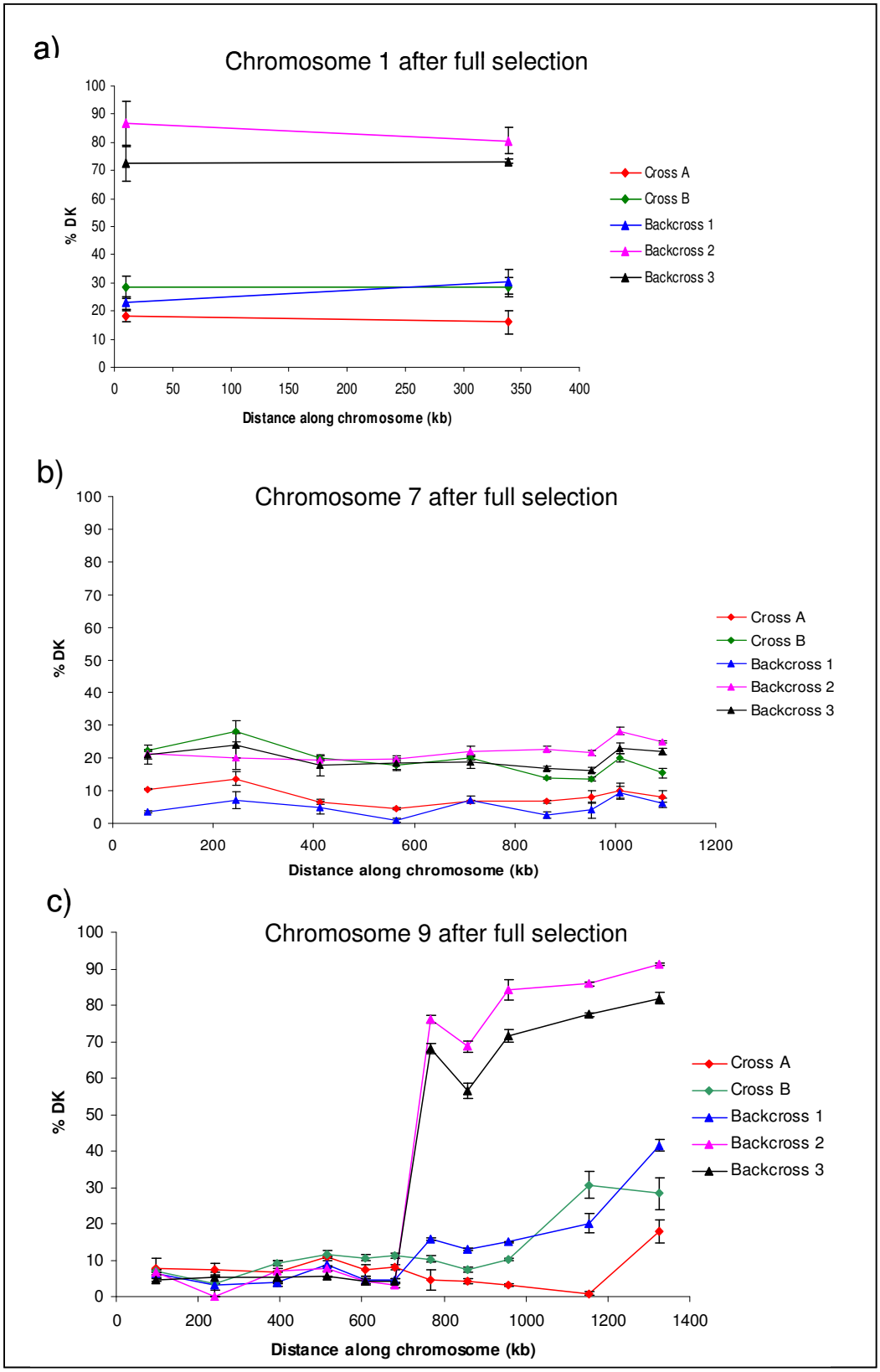
1 levels of DK for cross B, but a dramatic decrease from 75%-85% for backcrosses 2 and 3 (Figure 5.1a). Despite differences in the level of DK marker reduction between crosses and backcrosses, within each cross or backcross the % DK marker inheritance of all the chromosome 7 DK markers was fairly constant, with no particular DK marker being very strongly reduced.

The effects on DK markers across chromosome 9 after full fast-growth selection were quite striking. The first 6 DK markers, between 0kb and 700kb along the chromosome, were found in less than or equal to 10% of progeny from all five crosses and backcrosses (Figure 5.1c). Strong reduction was also seen across most of the rest of chromosome 9 for crosses A and B and backcross 1, with levels of DK inheritance only returning to that seen on chromosome 1, at the far right hand side of chromosome 9 (c.f. Figures 5.1a and 5.1c). However, in backcrosses 2 and 3, DK markers on the right hand side of chromosome 9 (750kb to 1400kb) were not reduced at all by growth selection (c.f. Figures 5.1a and 5.1c). In these two backcrosses there was a sudden increase in DK marker inheritance from around 5% to 70% between the 6^{th} and 7^{th} markers on chromosome 9 (between 700kb to 750kb along the chromosome).

5.3.3. Creation of Crosses C, D and E and Selection for Fast Growth

The region that appeared to be under strong selection on DK chromosome 9 was approximately 700kb in length (from 0kb – 700kb along the chromosome) and

Figure 5.1
The proportion of the cross progeny inheriting DK at (a) 2 marker positions on chromosome 1, (b) 9 marker positions on chromosome 7 and (c) 11 marker positions on chromosome 9, in cross progeny from crosses A, B and backcrosses 1, 2 and 3, after the final round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



therefore new crosses between DS and DK were made in order to try and narrow this region down. As described above, on one occasion DS and DK were crossed to give cross C, and then around six months later, a further two crosses were made, giving crosses D and E. Since less parasite DNA is required for pyrosequencing compared to AFLP marker analysis, only one mouse was required to grow the parasites for each round of selection for fast growth. Using the proportion of DK present in oocysts, a more accurate expectation of the number of recombinants in each cross could be made (see section 5.2.8 above) and was calculated as over 11,000 recombinants for cross C (Table 5.1). However, crosses D and E had fewer recombinants, 3620 and 537 respectively, than cross C because DK had transmitted significantly better than DS on these occasions (Table 5.1).

Sporozoites from cross C were inoculated into two mice, and when they reached 1% and 0.18% parasitaemia respectively on day 4 of infection, blood from both animals was pooled for passage for growth selection (Figure 5.2a). As before, at each round, 10^6 pRBCs were inoculated. The parasite population grew slowly over the first round of passage, but by the second and third rounds of passage, parasitaemias were almost 50% by day 8 and therefore, a fast-growing population had been selected (Figure 5.2a). Sporozoites from crosses D and E were inoculated into mice and the resulting sporozoite-induced infections from each cross were sub-inoculated for the first round of selection at <2% parasitaemia.

To establish the first and second rounds of growth selection, 10^6 pRBCs were

Table 5.1

The transmission success and numbers of recombinant cross progeny of crosses C, D and E. Crosses C, D and E were generated by mosquitoes taking a blood meal on day 6 of infection from two mice per cross, initially infected with a 1:5 mixture of DS:DK, a 1:1 mixture of DS:DK and a 1:2 mixture of DS:DK respectively.

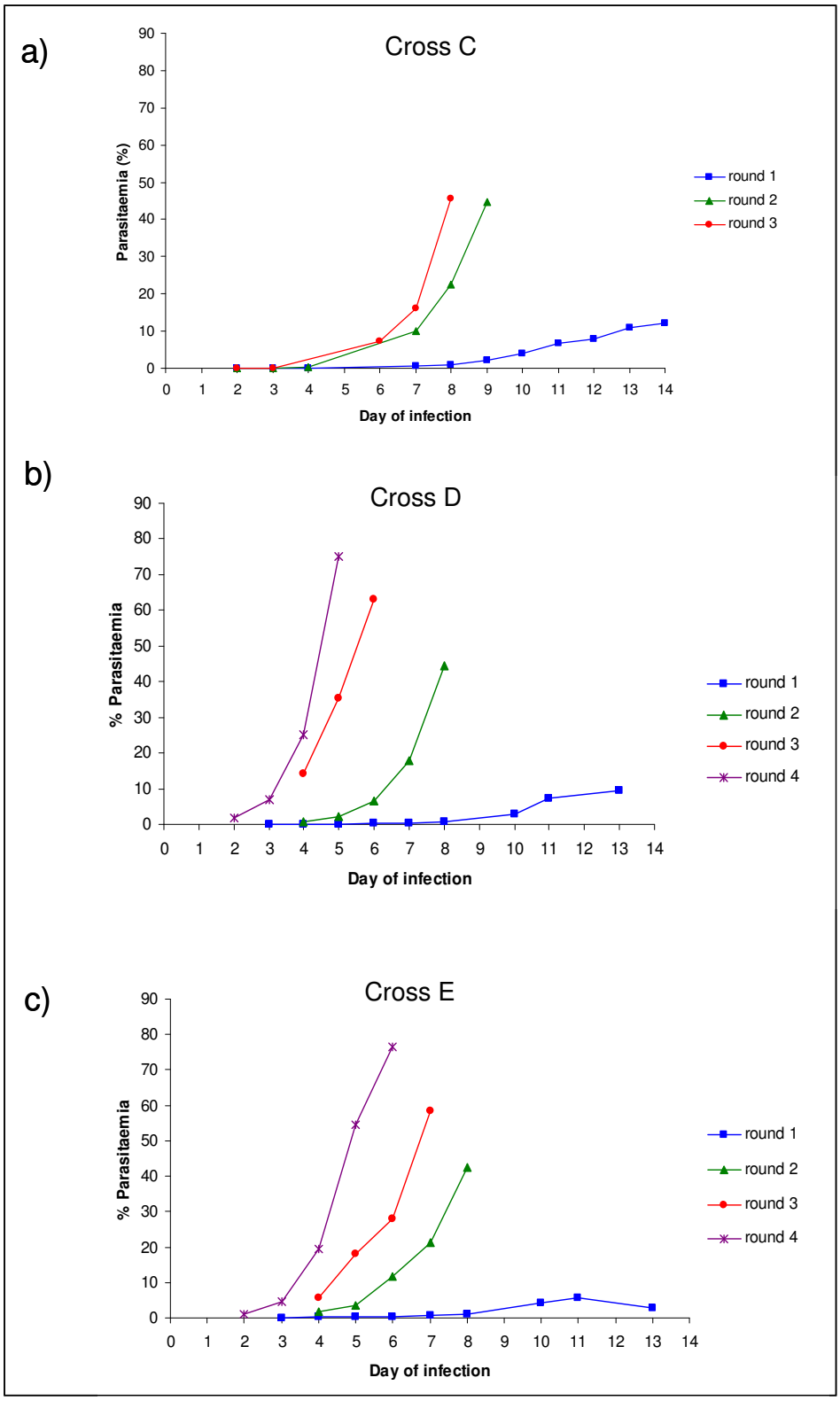
Cross progeny name	Mosquitoes infected % (mean oocysts / infected gut)	Number of mosquitoes dissected	Estimated total dissected mosquito oocyst burden	Number of meiotic products (proportion recombinants)	Theoretical maximum number recombinants
Cross C	93 (52.8)	128	6285	25141 (0.45)	11314
Cross D	85.7 (40)	88	3017	12067 (0.3)	3620
Cross E	91.7 (21)	109	2099	8396 (0.064)	537

inoculated (Figure 5.2b and 5.2c). Due to a holiday, stabilates were deep frozen after the second round of selection and then later taken out and inoculated for the third round of selection. This was subsequently repeated with the parasite populations being frozen down again after the third round and later inoculated for a final fourth round of growth selection. Therefore, each mouse was likely to have received more than 10^6 pRBCs for the third and fourth rounds of selection. The fourth round of selection was undertaken to ascertain if there was any difference in marker inheritance patterns with an extra round of selection, however no difference was found (data not shown). The growth characteristics of crosses D and E were similar, being initially slow and then extraordinarily fast by the 4^{th} round of selection (Figures 5.2b and 5.2c). However this was most likely due to the inoculation of much higher parasite doses in the third and final rounds of selection.

5.3.4. Effects on Chromosome 1, 7 and 9 DK Markers After Full Growth Selection of Crosses C, D and E

The inheritance of the same chromosome 1, 7 and 9 DK markers as used above in section 5.3.2 were tested in fully growth-selected cross C progeny (after 3^{rd} round of selection) and cross D and E progeny (after 4^{th} round of selection) (Figure 5.3). The background level of DK in the cross C progeny, as measured at the two markers on chromosome 1, was around 50% (Figure 5.3a). This was reduced in cross C right across chromosome 7 to around 10-20%, indicating quite strong selection acting on the whole of the chromosome, but with no particular marker reduced below 10% (Figure 5.3b). The second DK marker on chromosome 7 seemed to be the least

Figure 5.2
Selection of cross C, D and E progeny for fast growth over three or four rounds of blood passage. Each line represents the parasitaemia of an individual mouse. For cross C growth selection, 10^6 pRBCs were taken for passage at each stage. For crosses D and E, 10^6 pRBCs were taken for passage for the 1st and 2nd rounds of selection. For the 3rd and 4th rounds of selection, cross progeny stabilates, from the 2nd and 3rd rounds of selection respectively, were removed from deep freeze storage and inoculated directly into mice.



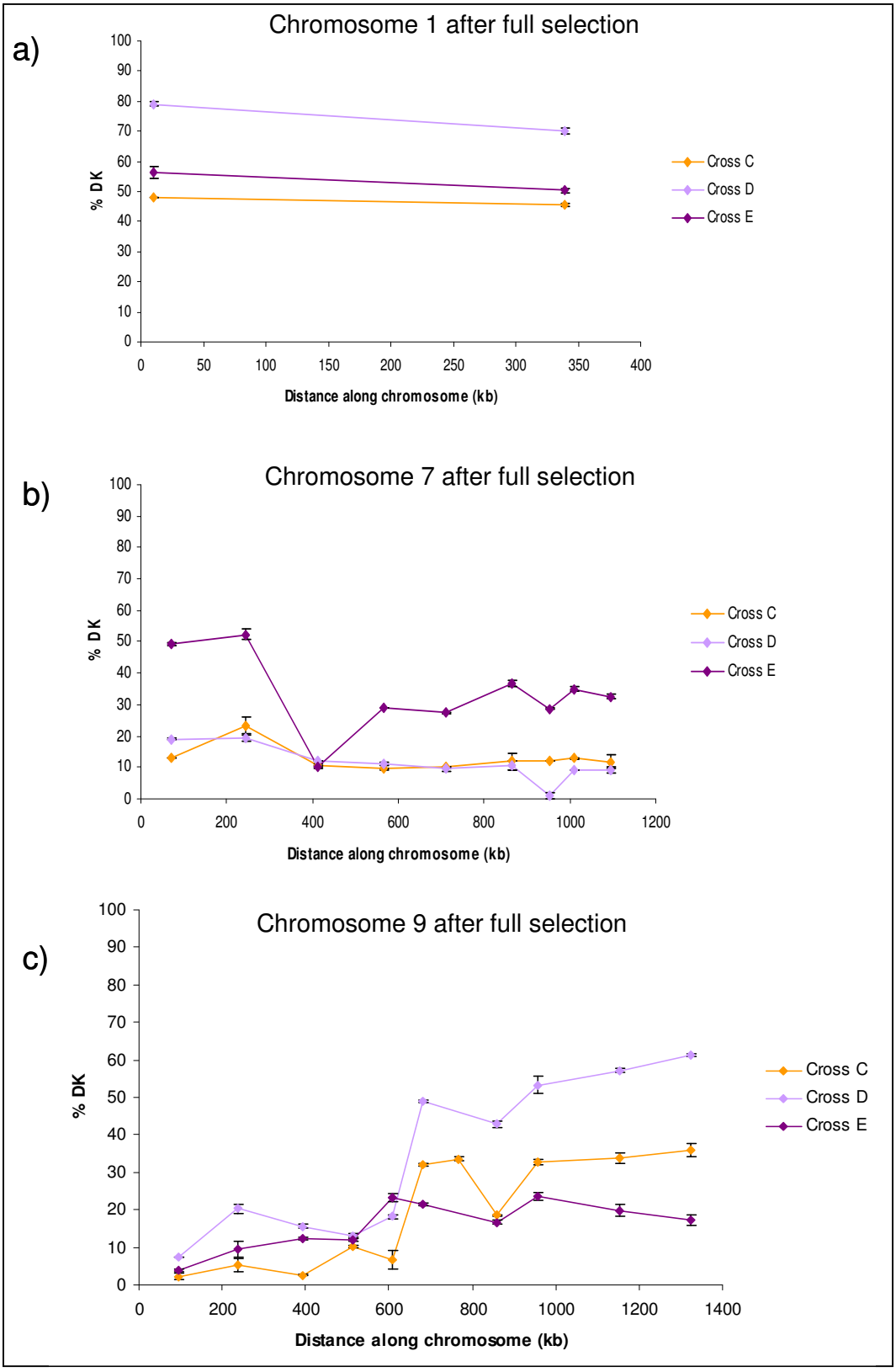
affected by selection, but even it was reduced to around 20%. The background level of DK was around 80% in cross D progeny and around 60% in cross E progeny (Figure 5.3a). DK markers across chromosome 7 were reduced to below 20% in cross D, with the 7th marker at around 900kb along chromosome 7 reduced to almost zero (Figure 5.3b). There was less chromosome 7 marker reduction in cross E, with almost no reduction of the first two markers, but a significant decrease to 10% for the 3rd marker at around 400kb.

However, the chromosome 9 marker patterns were much more distinct (Figure 5.3c). In cross C, the first five DK markers on chromosome 9 (0-600kb along the chromosome) were reduced to less than 10%, with the first three markers particularly strongly reduced by selection for fast growth. At the markers between 700kb and 1400kb along the chromosome, 30-40% DK inheritance was seen, which was close to the neutral 50% DK value seen for chromosome 1. For crosses D and E, chromosome 9 marker patterns also revealed that most DK marker reduction was on the left-hand side of the chromosome, with the first marker being the most reduced in both crosses (Figure 5.3c).

5.3.5. Comparison of the Effects on Chromosome 1, 7 and 9 DK Markers After Full Selection for Fast Growth of Crosses A, B, C, D and E and Backcrosses 1, 2 and 3

Putting all the DK marker inheritance data together from all the crosses and backcrosses examined in this chapter gives an overall picture of the robustness of marker behaviour under independent crossing and selection events (Figures 5.4 and

Figure 5.3
The proportion of cross C, D and E progeny inheriting DK at (a) 2 marker positions on chromosome 1, (b) 9 marker positions on chromosome 7 and (c)11 marker positions on chromosome 9, after the final round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



5.5). Cross progeny with background DK levels of between 15% and 90% have been examined (Figure 5.4a). It was clear that DK markers on chromosome 7 and 9 were affected following growth selection (Figures 5.4b, 5.4c and 5.5), in accordance with the AFLP marker analysis in Chapter 4.

The reduction of DK markers across chromosome 7 was usually across the whole chromosome and to the same extent for each cross or backcross, indicating that the effects of selection were not limited to one region of this chromosome in particular (Figure 5.4b and 5.5a). The level of reduction of DK markers on chromosome 7 varied between different crosses but DK marker inheritance was never consistently reduced to zero. Therefore, recombinant progeny with specific regions of DK chromosome 7 were never lost entirely from the cross progeny pool.

In contrast, on chromosome 9, there was a strong reduction of DK markers across a large region at the left-hand side of the chromosome in between markers 1 and 4 (Figures 5.4c and 5.5b). The levels of DK at the first three markers were not significantly different to each other (non-parametric Mann-Whitney *U*-test $p>0.26$). However, the level of DK at the first two markers was significantly less than that at the fourth marker, and there was a strong trend in this direction for the third marker (non-parametric Mann-Whitney *U*-test marker 1 v marker 4 $p<0.01$, marker 2 v marker 4 $p=0.02$, marker 3 v marker 4 $p=0.06$).

There was a large variation between crosses and backcrosses in the extent of DK marker inheritance in the remainder of chromosome 9. In backcrosses 2 and 3 and

Figure 5.4
The proportion of the cross progeny inheriting DK at (a) 2 marker positions on chromosome 1, (b) 9 marker positions on chromosome 7 and (c) 11 marker positions on chromosome 9, in cross progeny from crosses A, B, C, D and E and backcrosses 1, 2 and 3, after the final round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.

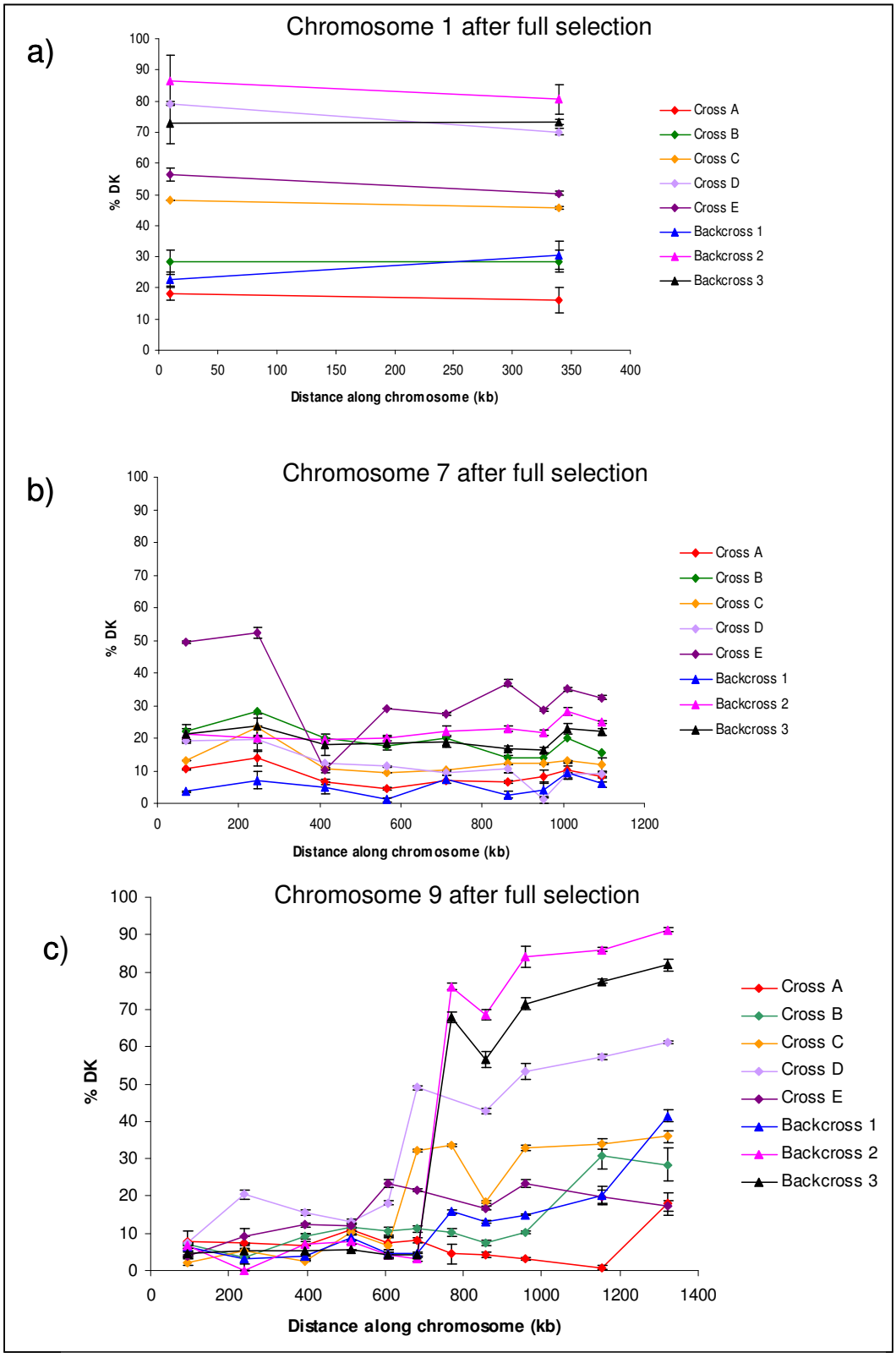
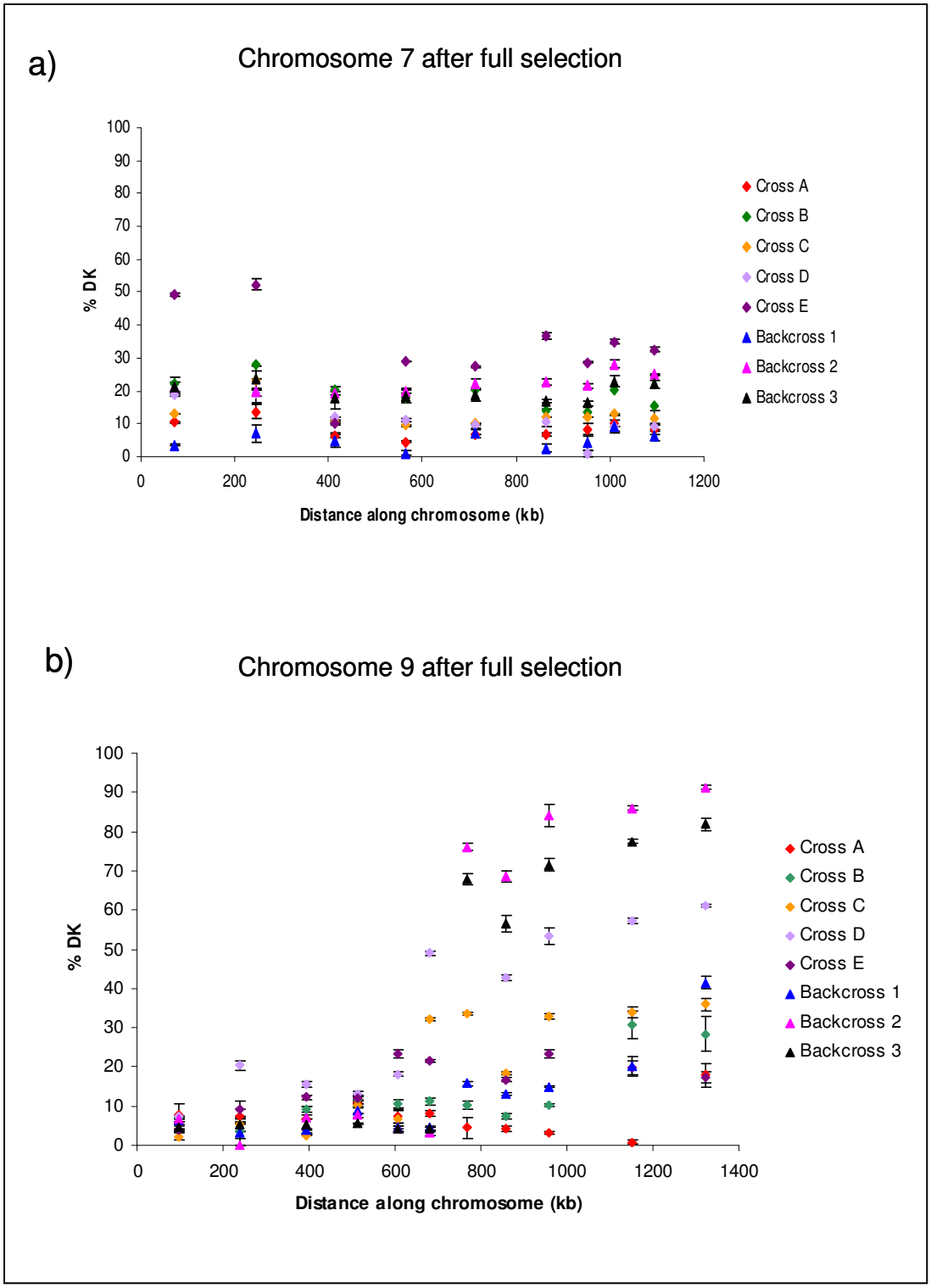


Figure 5.5
The proportion of the cross progeny inheriting DK at (a) 9 marker positions on chromosome 7 and (b) 11 marker positions on chromosome 9, in cross progeny from crosses A, B, C, D and E and backcrosses 1, 2 and 3, after the final round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



crosses C and D, there was a moderate reduction of the 8th DK chromosome 9 marker, which is around 900kb along the chromosome. However this did not fit into the overall impression of a lack of selection on the right hand side of chromosome 9 and could indicate that the real location of this marker is further towards the left-hand side. Marker locations were based on the *P. c. chabaudi* AS genome sequence and it may be possible that a rearrangement of this region has occurred in *P. c. adami*. In summary, of the chromosomes analysed in detail, the genetic region that appears to have the strongest effect on growth is to the left of marker 4 on chromosome 9.

5.3.6. Genes in Region of Interest on Chromosome 9

The region of the *P. chabaudi* genome between the left-hand end of chromosome 9 and the third marker is a minimum 376kb in length and contains approximately 89 genes (Table 5.2). This is an estimate because there may also be a further region of sequence comprising the subtelomeric region, and which is of undetermined size, upstream of the first gene in Table 5.2 (PC108181.00.0). This is because gaps between contigs in the *P. chabaudi* genome still remain to be closed, and if the contig does not contain genes for which an orthologue is present in *P. falciparum* (as is likely in a sub-telomeric region), the contig cannot be located in the *P. chabaudi* genome. The majority of the *P. chabaudi* genes in this region are conserved hypothetical proteins; however 83 of these genes have orthologues in *P. falciparum* and thus, can be described in greater detail based on their orthologue annotation (Table 5.2). However, even using the annotation from the *P. falciparum* genome, 43

(48%) of the 89 *P. chabaudi* genes are simply "hypothetical proteins" or "conserved *Plasmodium* proteins" for which no putative function has been assigned.

Further information can be gained by assessing the Gene Ontology terms for biological processes associated with each *P. falciparum* orthologous gene. The Gene Ontology is a standardised terminology used to describe the particular attributes of a gene product, and in this case, those applicable to biological processes (rather than cellular components or molecular functions) were used. Thirty-five (42%) of the 83 genes with *P. falciparum* orthologues could not be assigned a Gene Ontology term.

It is clear that the genes in this region of chromosome 9 are involved in a wide range of processes from transcription and translation to DNA repair and recombination, and protein folding. To assess whether there were a number of genes with similar functions in this region, the online MADIBA software program was used to test those genes that had an annotation and/or a gene ontology term(s) assigned to them (Law et al. 2008) (program online at http://www.bi.up.ac.za/MADIBA/) . This analysis showed that the numbers of these genes found in any particular metabolic pathway were no more than would be expected by chance. Therefore, there was no clustering of metabolic function in this region. Similarly, in general, there was no significant clustering by biological process of the genes in this region. The exceptions were two genes involved in signal transduction (orthologues of PF11_0050 and PF11_0147) and two genes involved in DNA recombination (orthologues of PF11_0071 and PF11_0087) which were both significant at the level of $0.0001 < p \leq 0.01$.

Table 5.2
P. chabaudi genes, with their annotated *P. falciparum* orthologues (if present), in the region under selection on *P. chabaudi* chromosome 9 (using information from the synteny map of (Kooij et al. 2005) Gene order progresses from the far left-hand end of chromosome 9 (top of the table), to the third DK marker at around 400kb along chromosome 9 (bottom of table). *P. chabaudi* genes in which markers fall are highlighted in red. Gene annotation and gene ontology terms were assigned using PlasmoDB, release 5.4 (http://plasmodb.org/plasmo/).

P.chabaudi gene	*P.chabaudi* gene annotation	Orthologous *P. falciparum* gene	*P. falciparum* gene annotation	Gene ontology terms (Biological processes) /further information
PC108181.00.0	Pc-fam2 protein, putative	***none***		
PC109406.00.0	Pc-fam5 protein, putative	***none***		
PC100370.00.0	Pc-fam5 protein, putative	***none***		
PC403354.00.0	hypothetical protein	***none***		
PC402498.00.0	hypothetical protein	***none***		
PC402492.00.0	hypothetical protein	***none***		
PC000339.02.0	conserved hypothetical protein	PF11_0044	conserved Plasmodium protein	
PC000340.02.0	conserved hypothetical protein	PF11_0045	CPW-WPC family	proteins with motifs of CPxxW and (less well conserved) WPC - unknown function
PC000799.00.0	conserved hypothetical protein	PF11_0047	actin-related protein, putative	protein binding
PC000539.01.0	casein kinase II beta chain, putative	PF11_0048	casein kinase II beta chain, putative	protein amino acid phosphorylation
PC301146.00.0	conserved hypothetical protein	PF11_0049	conserved plasmodium protein/Not1 domain containing protein	Not1 forms a complex with Ccr4 to form a transcriptional regulator
PC000143.00.0	conserved hypothetical protein	PF11_0050	hypothetical protein	signal transduction
PC000595.01.0	phenylalanine -- tRNA ligase, putative	PF11_0051	phenylalanine -- tRNA synthetase beta chain, putative	phenylalanine-tRNA aminoacylation
PC000597.01.0	conserved hypothetical protein	PF11_0052	syntaxin, putative	Membrane integrated Q-SNARE protein participating in exocytosis.
PC000596.01.0	PfSNF2L, putative	PF11_0053	PfSNF2L	chromatin remodelling and transcriptional regulation
PC000093.04.0	conserved hypothetical protein	PF11_0054	hypothetical protein	
PC000021.02.0	conserved hypothetical protein	PF11_0055	conserved Plasmodium protein, conserved in alveolata	
PC000022.02.0	conserved hypothetical protein	PF11_0056	conserved Plasmodium protein, unknown function	
PC000023.02.0	conserved hypothetical protein	PF11_0057	conserved Plasmodium protein, unknown function	
PC000024.02.0	conserved hypothetical protein	PF11_0058	RNA polymerase subunit, putative	transcription

P.chabaudi gene	*P.chabaudi* gene annotation	Orthologous *P. falciparum* gene	*P. falciparum* gene annotation	Gene ontology terms (Biological processes) / further information
PC300119.00.0	conserved hypothetical protein	PF11_0059	metabolite/drug transporter	
PC000063.04.0	protein kinase, putative	PF11_0060	Calcium/calmodulin-dependant protein kinase, putative	protein amino acid phosphorylation
PC302253.00.0	histone H2B, putative	PF11_0062	histone H2B	nucleosome assembly and chromosome organisation
PC000224.01.0	conserved hypothetical protein	PF11_0063	hypothetical protein	
PC000332.02.0	conserved hypothetical protein	PF11_0064	hypothetical protein	
PC000415.04.0	**ribosomal protein S4, putative**	**PF11_0065**	**40S ribosomal protein S4, putative - marker 1**	**translation**
PC000517.01.0	caltractin (centrin), putative	PF11_0066	caltractin (centrin), putative	mitosis - centriole duplication
PC000864.03.0	conserved hypothetical protein	PF11_0067	hypothetical protein	
PC000172.03.0	conserved hypothetical protein	PF11_0068	conserved Plasmodium protein, unknown function	
PC000652.04.0	conserved hypothetical protein	PF11_0069	hypothetical protein	
PC300721.00.0	RuvB DNA helicase, putative	PF11_0071	RuvB DNA helicase, putative	DNA repair/recombination
PC000043.01.0	conserved hypothetical protein	PF11_0072	apicoplast ribosomal protein S15 precursor, putative	translation
PC000042.01.0	conserved hypothetical protein	PF11_0073	conserved Plasmodium protein, unknown function	
PC000370.01.0	conserved hypothetical protein	PF11_0074	exonuclease, putative	intein-mediated protein splicing
PC301990.00.0	conserved hypothetical protein	PF11_0075	hypothetical protein	
PC000675.00.0	conserved hypothetical protein	PF11_0076	hypothetical protein	
PC000086.00.0	conserved hypothetical protein	PF11_0077	DEAD/DEAH-box helicase domain containing protein, conserved Plasmodium protein	nucleic acid and ATP binding and helicase activity
PC001320.02.0	conserved hypothetical protein	PF11_0078	hypothetical protein	
PC001321.02.0	conserved hypothetical protein	PF11_0079	hypothetical protein	protein amino acid phosphorylation
PC300135.00.0	hypothetical protein	PF11_0083	nucleic acid binding factor, putative	
PC000703.01.0	conserved hypothetical protein	PF11_0084	hypothetical protein	
PC000829.01.0	conserved hypothetical protein	PF11_0086	MIF4G domain containing protein	RNA binding

P.chabaudi gene	*P.chabaudi* gene annotation	Orthologous *P. falciparum* gene	*P. falciparum* gene annotation	Gene ontology terms (Biological processes) / further information
PC000445.01.0	Rad51 homolog, putative	PF11_0087	Rad51 homolog, putative	DNA repair/recombination
PC000841.00.0	conserved hypothetical protein	PF11_0088	hypothetical protein	
PC000402.01.0	hypothetical protein	PF11_0089	hypothetical protein	
PC300579.00.0	conserved hypothetical protein	PF11_0090	nucleolar rRNA processing protein, putative	ribosome biogenesis and assembly, and cell proliferation
PC301184.00.0	conserved hypothetical protein	PF11_0091	transcription factor with AP2 domains, putative	
PC000804.04.0	conserved hypothetical protein	PF11_0092	mechanosensitive ion channel protein	
PC300903.00.0	conserved hypothetical protein	PF11_0093	IWS1-like protein, putative	IWS1 is an RNA polymerase II transcription elongation factor
PC000044.04.0	conserved hypothetical protein	PF11_0094	hypothetical protein	
PC000043.04.0	conserved hypothetical protein	PF11_0095	histidyl-tRNA synthetase	D-amino acid catabolic process
PC000095.02.0	succinyl-CoA synthetase alpha subunit, putative	PF11_0097	succinyl-CoA synthetase alpha subunit, putative	tricarboxylic acid cycle (TCA cycle)
PC000827.03.0	endoplasmic reticulum-resident calcium binding protein, putative	PF11_0098	endoplasmic reticulum-resident calcium binding protein	intra-cellular protein transport
PC301594.00.0	heat shock protein DnaJ homologue Pfj2, putative	PF11_0099	heat shock protein DnaJ homologue Pfj2	protein folding and response to heat
PC000631.04.0	conserved hypothetical protein	PF11_0100	hypothetical protein	
PC000630.04.0	**conserved hypothetical protein**	**PF11_0101**	**hypothetical protein – marker 2**	
PC000192.02.0	conserved hypothetical protein	PF11_0540	hypothetical protein	
PC000638.01.0	conserved hypothetical protein	PF11_0105	conserved protein, unknown function	
PC000701.03.0	conserved hypothetical protein	PF11_0107	hypothetical protein	
PC000517.02.0	conserved hypothetical protein	PF11_0108	U5 snRNP associated protein, putative	RNA processing
PC000518.02.0	conserved hypothetical protein	PF11_0109	hypothetical protein	
PC000716.02.0	hypothetical protein	PF11_0111	asparagine-rich antigen	nucleic acid binding
PC000398.02.0	vacuolar sorting protein 35, putative	PF11_0112	vacuolar sorting protein 35, putative	retrograde transport from endosome to golgi

P.chabaudi gene	*P.chabaudi* gene annotation	Orthologous *P. falciparum* gene	*P. falciparum* gene annotation	Gene ontology terms (Biological processes) /further information
PC000399.02.0	ribosomal protein L11, putative	PF11_0113	mitochondrial ribosomal protein L11, putative	translation
PC000793.04.0	actin, putative	PF11_0114	actin-like protein homolog, ALP1 homolog	cytoskeleton organisation and biogenesis
PC000912.01.0	conserved hypothetical protein	PF11_0115	hypothetical protein	
PC001395.02.0	conserved hypothetical protein	PF11_0116	hypothetical protein	
PC001394.02.0	replication factor C subunit 5, putative	PF11_0117	replication factor C subunit 5, putative	DNA strand elongation during DNA replication
PC001396.02.0	conserved hypothetical protein	PF11_0118	conserved protein	
PC302002.00.0	conserved hypothetical protein	PF11_0120	hypothetical protein	
PC000585.02.0	conserved hypothetical protein	PF11_0122	endonuclease/exonuclease/phosphatase domain containing protein	
PC000109.04.0	conserved hypothetical protein	PF11_0123	hypothetical protein	
PC000108.04.0	conserved hypothetical protein	PF11_0124	conserved protein, unknown function	
PC000538.04.0	conserved hypothetical protein	PF11_0125	hypothetical protein	
PC000845.03.0	conserved hypothetical protein	PF11_0127	hypothetical protein	protein amino acid phosphorylation
PC000176.04.0	conserved hypothetical protein	PF11_0128	coq4 homolog, putative	Coenzyme Q (ubiquinone) biosynthesis protein
PC000175.04.0	conserved hypothetical protein	PF11_0129	hypothetical protein	
PC000254.03.0	conserved hypothetical protein	PF11_0526	hypothetical protein	
PC000253.03.0	conserved hypothetical protein	PF11_0131	hypothetical protein	
PC302219.00.0	conserved hypothetical protein	PF11_0527	conserved Plasmodium protein, unknown function	
PC302272.00.0	conserved hypothetical protein	PF11_0528	hypothetical protein	translation and proteolysis and pathogenesis (Botulinum toxin-like domain)
PC000676.00.0	protein tyrosine phosphatase, putative	PF11_0139	protein tyrosine phosphatase, putative	dephosphorylation
PC000722.01.0	UDP-galactose transporter, putative	PF11_0141	UDP-galactose transporter, putative	
PC000040.01.0	conserved hypothetical protein	PF11_0142	Ubiquitin-domain containing protein	protein modification
PC000388.03.0	conserved hypothetical protein	PF11_0143	Pf-GTP-binding protein	

P.chabaudi gene	*P.chabaudi* gene annotation	Orthologous *P. falciparum* gene	*P. falciparum* gene annotation	Gene ontology terms (Biological processes) / further information
PC000518.04.0	conserved hypothetical protein	PF11_0144	Rpr2, RNAse P, putative	
PC000516.04.0	glyoxalase I, putative	PF11_0145	glyoxalase I	methylglyoxalase metabolic processing
PC302215.00.0	conserved hypothetical protein	PF11_0146	hypothetical protein	
PC001036.02.0	**mitogen-activated protein kinase 2, putative**	**PF11_0147**	**mitogen-activated protein kinase 2 - marker 3**	**protein amino acid phosphorylation, MAPKKK cascade signal transduction**

5.4. Discussion

This chapter investigated DK marker inheritance in the original crosses A and B, and backcrosses 1, 2 and 3 from Chapter 4, in addition to that in three new crosses, C, D and E. By examining the inheritance of 9 DK markers on chromosome 7 and 11 DK markers on chromosome 9, and comparing them to neutral DK markers on chromosome 1, the general pattern established by the AFLP marker inheritance patterns in Chapter 4 was confirmed. The three reduced DK AFLP markers on chromosome 7 had been located widely across the chromosome. When chromosome 7 was looked at in detail using pyrosequencing markers, it appeared that selection was indeed acting right across the whole chromosome. The one reduced DK AFLP marker on chromosome 9 had been located on the left-hand side of the chromosome. On examining the whole of chromosome 9 at approximately 100-200kb intervals, DK markers on the left-hand side were also found to be consistently strongly reduced, whereas those on the right hand side of the chromosome were not.

The region that appeared to be affected by selection on chromosome 9 included DK markers 1 to 6 in the initial crosses A and B, and backcrosses 1, 2 and 3. However, with the creation of crosses C, D and E, the region consistently affected by selection on chromosome 9 comprised only DK markers 1 to 3 and therefore, was reduced from approximately 700kb to 400kb. This was presumably due to the selection of parasites from the cross C, D and E progeny pools in which different recombination events had occurred. This clearly demonstrated very strong selection against cross progeny carrying the first three DK markers on chromosome 9 in all pools of recombinant progeny tested, while there were varying degrees of DK marker

inheritance in the remainder of the chromosome. This indicates the presence on chromosome 9 of a gene (or genes) with a major effect on the control of parasite growth.

In contrast, no obvious region of DK chromosome 7 was consistently selected against; rather, there was a general reduction in inheritance of the whole of DK chromosome 7. This could be consistent with, for example, the existence of several DS chromosome 7-wide loci which are moderately important for determining growth characteristics but which can be inherited in an "either / or / and" fashion and still confer the fast-growth phenotype.

Therefore, the region of the *P. chabaudi* genome which is of most interest to the study of growth determinants, is the region of chromosome 9 between the start of the chromosome and marker 3. This section is approximately 376kb in length, excluding the subtelomeric region (which is of unknown length). This region contains approximately 89 genes, 43 of which are hypothetical or conserved *Plasmodium* proteins with no putative function assigned to an orthologue in *P. falciparum*. The genes which have been annotated are involved in many different cellular processes e.g. transcription, translation, phosphorylation, and many others. As growth characteristics could be determined by any number of different biological processes, it is difficult to locate the most likely candidate gene(s) in such a long list. Analysis of Gene Ontology terms did not reveal significant numbers of genes in this region which were part of the same metabolic pathway or biological process, apart from signal transduction and DNA recombination. However, nearly half of the genes in

this region could not be assigned Gene Ontology terms and this therefore, limits the power of such an analysis.

In addition to the many candidate genes in this region of chromosome 9, genes in the subtelomeric region to the left hand side of marker 1 must also be considered, although due to the incomplete *P. chabaudi* genome assembly, its sequence is not known. The subtelomeric regions of *P. chabaudi* are known to contain many copies of variant multi-gene family genes (Fischer et al. 2003). The subtelomeric structure is a mosaic made up of telomeric repeats and intergenic repeats interspersed with multi-gene family genes including: *pyst-a, pyst-b* and *pyst-c1* homologues, *cir* genes, *cir*-like genes, hydrolases, *etramp* genes, other unknown *P. chabaudi* specific gene family members as well as unique single copy *P. chabaudi* genes of unknown function (Fischer et al. 2003). The *P. chabaudi cir* genes are members of a *Plasmodium* variant gene superfamily; the *pir* superfamily, which also includes the *rif* genes of *P. falciparum*, the *vir* genes of *P. vivax*, the *kir* genes of *P. knowlesi*, the *bir* genes of *P. berghei* and the *yir* genes of *P. yoelii* (Janssen et al. 2001; Janssen et al. 2004). There are an estimated 138 *cir* genes in the *P. chabaudi* genome (Hall et al. 2005) which are thought to be predominantly subtelomeric and whose gene products are expressed on the surface of infected erythrocytes (Janssen et al. 2004). Their exact function has not yet been elucidated, but due to their variant nature it has been suggested that they may be involved in immune evasion by antigenic variation.

From the LGS analysis of this chapter and Chapter 4, it is clear that the determination of growth characteristics in *P. c. adami* is not due to a single genetic

locus; although there is a major locus on chromosome 9, loci across chromosome 7 and perhaps on chromosome 6, are also involved. As will be described in detail below, there are several examples in the literature where *P. berghei* or *P. falciparum* genes have been disrupted or their proteins inhibited, and this has had a detrimental effect on parasite growth. These genes are involved in metabolism, invasion and other biological processes, therefore the many different genetic loci identified by the present LGS analysis might represent the location of genes involved in more than one of these processes.

For genes involved in metabolism, it is perhaps unsurprising that the loss of a "house-keeping gene" should decrease parasite growth; e.g. RNAi directed against the *P. falciparum dhodh* and *aroC* genes, involved in pyrimidine and folate biosynthesis respectively, significantly decreased *in vitro* growth of the parasite (McRobert and McConkey 2002). Therefore any number of the housekeeping genes in the region of interest on chromosome 9, or across chromosome 7, might differ between DS and DK, and perhaps lead to the accelerated growth of DS relative to DK, or the reduced growth of DK relative to DS.

For genes involved in invasion, it is clear that manipulation of invasion proteins does have a significant effect on parasite growth. From Chapter 2 it was clear that DS was able to invade a larger proportion of the erythrocyte pool than DK, however as this property did not change over time as the difference in parasitaemia did, it was unlikely to account entirely for the difference in growth between DS and DK. In *P. berghei*, disruption of the invasion protein merozoite surface protein 7 (*msp 7*) led to

a decrease in parasite growth over the first week of infection due to a decrease in invasion of mature erythrocytes (Tewari et al. 2005). Similarly, antibodies to the *P. yoelii* 235 protein prevented the fast-growing *P. yoelii* YM strain from invading normocytes and reaching lethally high parasitaemias (Holder and Freeman 1981). Finally, duplication of the genes encoding invasion proteins surfin$_{4.1}$ and PfRh1 was associated with the faster *in vitro* growth rate of *P. falciparum* laboratory isolates F32 and FCR3, relative to other isolates tested (Ribacke et al. 2007). Although orthologues of these genes are not present in the chromosome 9 region of interest in *P. chabaudi*, it is possible that another protein involved in invasion is encoded by a gene in this region and that this differs between DS and DK. Although such a gene on chromosome 9 is not obvious from the annotation shown in Table 5.2, there are a large number of genes with no assigned annotation in this region, and of course, a large number of such genes in the subtelomeric region.

Other properties have been associated with some *Plasmodium* parasites that possess higher multiplication rates than others. One is the ability of a parasite to rosette, i.e. the ability of a parasitised erythrocyte to surround itself with uninfected erythrocytes, *in vitro*. In splenectomised *Saimiri sciureus* monkeys (in which sequestration does not occur) a rosetting variant of the *P. falciparum* Palo Alto strain had a higher multiplication rate than a non-rosetting variant (Le Scanf et al. 2007). However, rosetting was not associated with multiplication rate in isolates from African children (Deans et al. 2006). *P. chabaudi* strains, including DS and DK, have been found to produce rosettes after short-term *in vitro* culture of trophozoite stages (Mackinnon et al. 2002). However, any differences in rosetting frequency between DS and DK were

not recorded and the parasite gene(s) which may be responsible for rosetting in *P. chabaudi* are unknown.

Secondly, there is some evidence that in parasites cultured *in vitro*, mutants arise which have lost their ability to produce gametocytes and these mutants grow faster, over-growing the remaining gametocyte-producing parasites. Several different karyotype mutants of *P. berghei* clone 8417 that did not produce gametocytes were generated by repeated mechanical passage in mice and these grew up to dominate the parasite population (Janse et al. 1992). However, between the *P. berghei* karyotype mutants there was no consistent change in the size of any particular chromosome which might indicate that a specific region was involved in gametocytogenesis and parasite growth. A subtelomeric deletion of part of *P. falciparum* chromosome 9 has also been associated with loss of asexual cytoadhesion and gametocytogenesis in *in vitro* culture of isolates (Day et al. 1993). These findings are interesting given that DS, although it has not lost its ability to produce gametocytes, does produce significantly fewer gametocytes than DK (see Chapter 2). However, chromosome 9 of *P. falciparum* is not syntenic with either chromosomes 6, 7 or 9 in *P. chabaudi*.

In conclusion, by taking a genetic analysis approach through linkage group selection, a region approximately 400kb in size has been found on *P. chabaudi* chromosome 9 in which is located a gene or genes which appear to have a major effect on parasite growth. There are a large number of potential candidate genes in this region, and none have been specifically associated with the control of growth in other malaria parasite studies. Therefore, further experiments must be carried out to narrow this

region down further and to determine the mechanism for the difference in growth between DS and DK. These are addressed in the general discussion to this thesis. Meanwhile, there is one last issue to address experimentally in this thesis, that of the timing within the parasite life cycle of the selection against cross progeny carrying DK markers in the region of interest on chromosome 9. The effects on DK markers on chromosomes 7 and 9 have been assessed in this chapter after several rounds of growth in the blood. However, since cross progeny arise first in the tissues of the mosquito, the possibility exists that the marker inheritance patterns seen at this stage are the result of selection occurring much earlier, either in the mosquito itself or in the liver stage of the lifecycle. These possibilities are explored in the experiments in Chapter 6.

Chapter 6: Investigation of the Stage of the Lifecycle in which Selection acts on Cross Progeny

6.1. Introduction

The LGS analysis of Chapters 4 and 5 had revealed that DK markers in a 400bp region of *P. chabaudi* chromosome 9 were consistently reduced in cross progeny fully selected for fast growth characteristics. However, as marker inheritance was only measured at the end of the final round of growth selection in the blood, it was possible that the selection on the cross progeny could have already occurred in the mosquito or liver stage of the lifecycle. Therefore, it was important to test whether the loss of recombinants with DK chromosome 9 markers had occurred before blood stage growth. This would determine if the genetic loci identified were definitely involved in the control of blood stage growth (and thus pathogenesis), rather than in the liver or mosquito.

As had been seen in Chapter 4, the AFLP marker patterns in crosses A and B, and backcrosses 1, 2 and 3 appeared to change very little over the course of successive blood infections, as DK markers which were strongly reduced by the third round of growth selection in the blood, were already reduced by the first round. This is confirmed in the present chapter by measuring pyrosequencing marker reduction over successive rounds of blood growth selection in these crosses and backcrosses. In addition, due to the sensitivity of pyrosequencing, oocyst and sporozoite DNA, and also parasite DNA from low-parasitaemia sporozoite-induced infections, could all now be used to assess DK marker inheritance at stages of the lifecycle prior to blood

stage growth. In the present chapter, analysis of oocyst and sporozoite material from cross C indicates that selection was not occurring in the mosquito. Moreover, further analysis of sporozoite-induced and initial blood infections of cross C, D and E progeny revealed that selection on chromosome 9 was, in fact, occurring only after a limited period of blood stage growth within the first week of selection for fast growth. These results validate the region of interest on chromosome 9 as the location for a gene or genes which have a major effect on the blood stage growth of the rodent malaria parasite *P. c. adami*.

6.2. Materials and Methods

The creation and selection of crosses A, B, C, D and E, and backcrosses 1, 2 and 3 for fast growth has already been described in Chapters 4 and 5. DNA was extracted from blood-stage infections and from oocysts as described in Chapter 5, section 5.2.2. For the purposes of the experiments in this chapter, during the growth of cross progeny, DNA was also extracted from daily blood spots (approximately 2µl blood), from mashed infected mosquito mid-guts and from crushed infected mosquito salivary glands, all of which were dried onto FTA Classic Card (Whatman). A single disc per sample was cut from the card with a 1.2mm diameter micropunch (Harris). Blood sample discs were washed with 200µl FTA reagent (Whatman) for 5 minutes, the supernatant was removed and this step was repeated. Lastly, the disc was washed with sterile MilliQ water for 5 minutes, before all liquid was removed. Oocyst and sporozoite sample discs were washed only once in 100µl FTA reagent before being washed with sterile MilliQ water for 5 minutes. Measurement of the inheritance of

DK markers by pyrosequencing was conducted on these DNA samples using protocols already described in Chapter 5, sections 5.2.3 and 5.2.4.

6.3. Results

6.3.1. Effects on DK Markers on Chromosome 7 and 9 over Three Rounds of Growth Selection in Crosses A and B and Backcrosses 1, 2 and 3

On chromosomes 7 and 9, four marker locations were chosen to examine the dynamics of selection on both chromosomes over three rounds of blood stage growth (Figure 6.1). All four chromosome 7 DK markers in cross A were strongly reduced to 5-10% by the first round of selection but there was no further reduction by subsequent rounds of selection (Figure 6.1a). However, there was a reduction in chromosome 7 DK marker inheritance with successive rounds of selection in the progenies of cross B, and backcrosses 1, 2 and 3 (Figure 6.1b-e). On chromosome 9 (Figure 6.2), marker inheritance in crosses A and B and backcross 2 changed little over the three rounds of selection, with only backcrosses 1 and 3 showing some change over successive rounds of selection. Therefore the strong reduction of three markers in the region of interest on the left hand side of chromosome 9 had already occurred by the end of the first round of selection.

Figure 6.1
The proportion of the cross progeny inheriting DK at 4 marker positions on chromosome 7 (markers 3, 6, 7 and 8 see Appendix 1) in the cross progeny from crosses A, B and backcrosses 1, 2 and 3, after the 1st, 2nd and 3rd rounds of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.

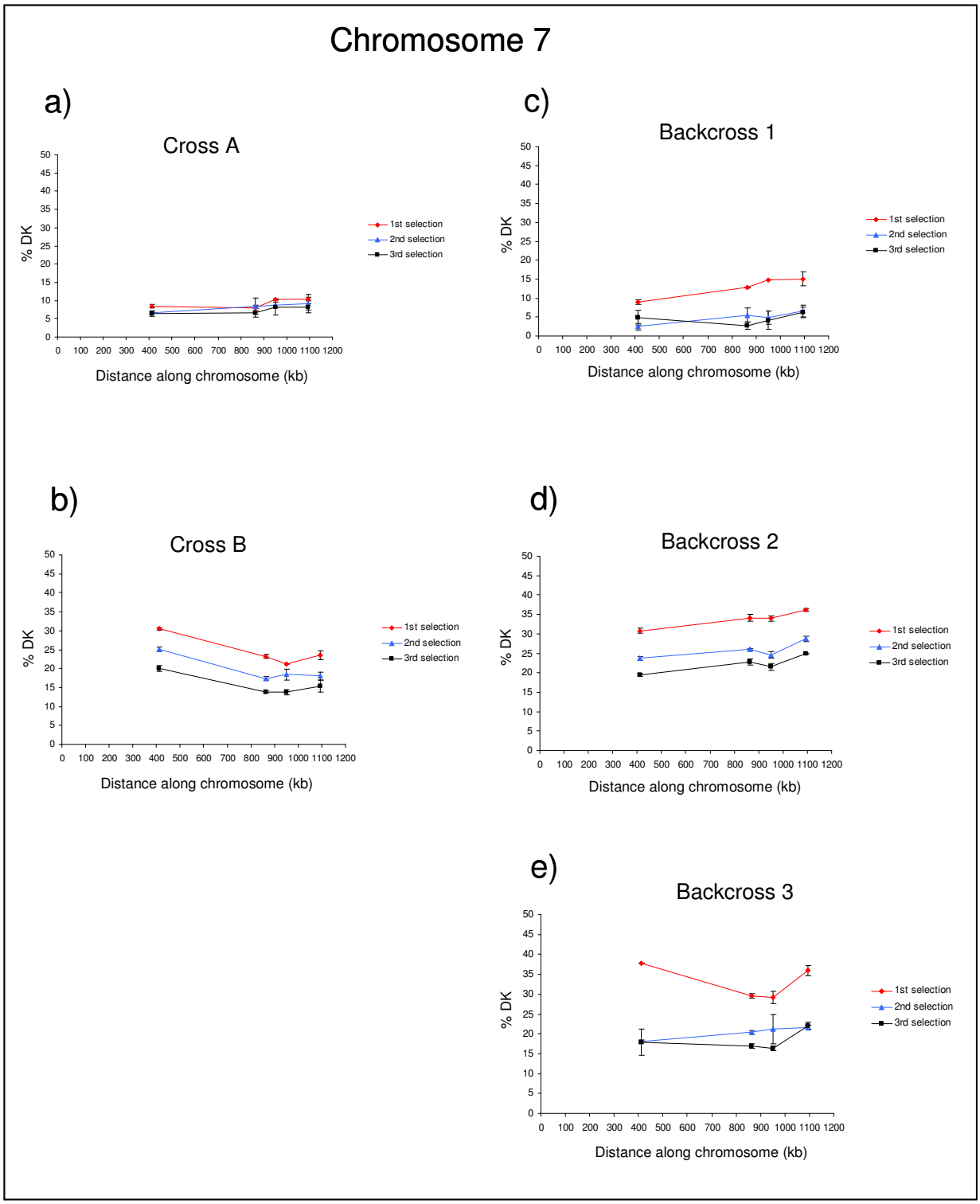
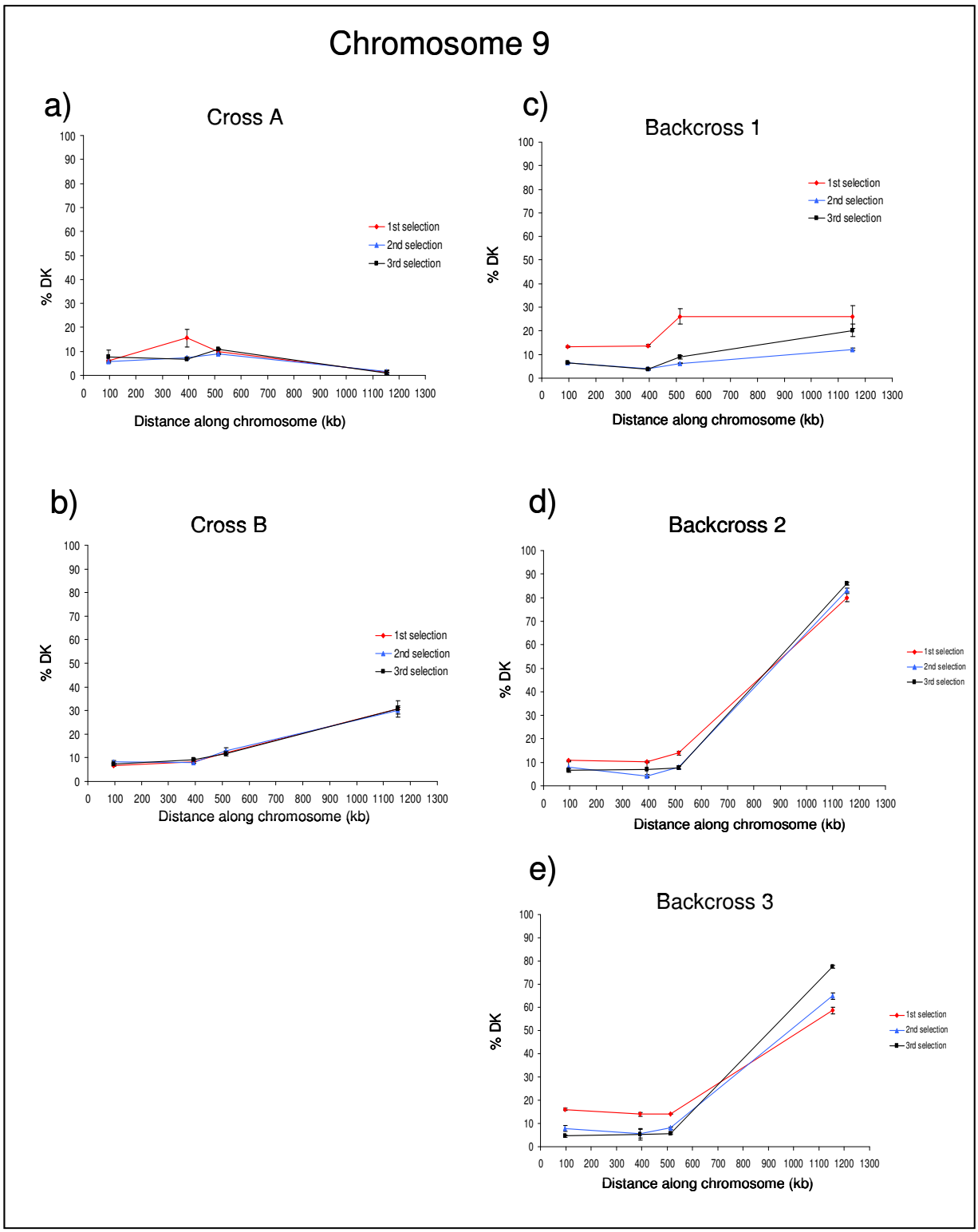


Figure 6.2
The proportion of the cross progeny inheriting DK at 4 marker positions on chromosome 9 (markers 1, 2, 4 and 10 see Appendix 1) in cross progeny from crosses A, B and backcrosses 1, 2 and 3, after the 1st, 2nd and 3rd rounds of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



6.3.2. Reduction of DK Markers on Chromosome 9 in Cross C Progeny at Different Stages of the Lifecycle

Using material from oocysts, sporozoites, and sporozoite-induced infections that had been collected for cross C, the inheritance of two DK markers on chromosome 9 **was** compared in each of these preparations (Figure 6.3). Due to the limited amount of material, only two markers were tested; one was the first marker on chromosome 9 which was in the region which was strongly reduced under selection in the blood (chromosome 9 marker 1, see Appendix 1) and the other was at the far right hand side of chromosome 9 in the region unaffected by selection (chromosome 9 marker 10, see Appendix 1). Both DK markers were inherited by between 60-80% of the cross progeny in the mosquito stages (oocyst and sporozoite) and in blood parasites shortly after emergence from the liver stage (sporozoite-induced infection). It was only after one round of growth in the blood (i.e. measured after 14 days of growth) that the amount of DK in the cross progeny decreased to 30% in the unaffected region of chromosome 9, and to less than 5% in the affected region.

6.3.3. Timing of Selection on Chromosome 9 during the First Round of Blood Stage Growth of Cross C Progeny

The first round of growth in the blood had taken 14 days for cross C, being particularly slow and only reaching a low parasitaemia of around 10% (Figure 6.4a). Small blood spot samples had been taken during this period and were now used to trace the dynamics of chromosome 9 selection (Figure 6.4a). Four chromosome 9 markers were measured; these were the first four on chromosome 9 (chromosome 9 markers 1, 2, 3 and 4, see Appendix 1) and therefore, in the region of interest. The

Figure 6.3
The proportion of cross C progeny inheriting DK at 2 marker positions on chromosome 9 (markers 1 and 10, see Appendix 1) when measured at the oocyst stage, sporozoite stage, after emergence in the blood following the sporozoite infection of the liver, and after the 1st, 2nd and 3rd rounds of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.

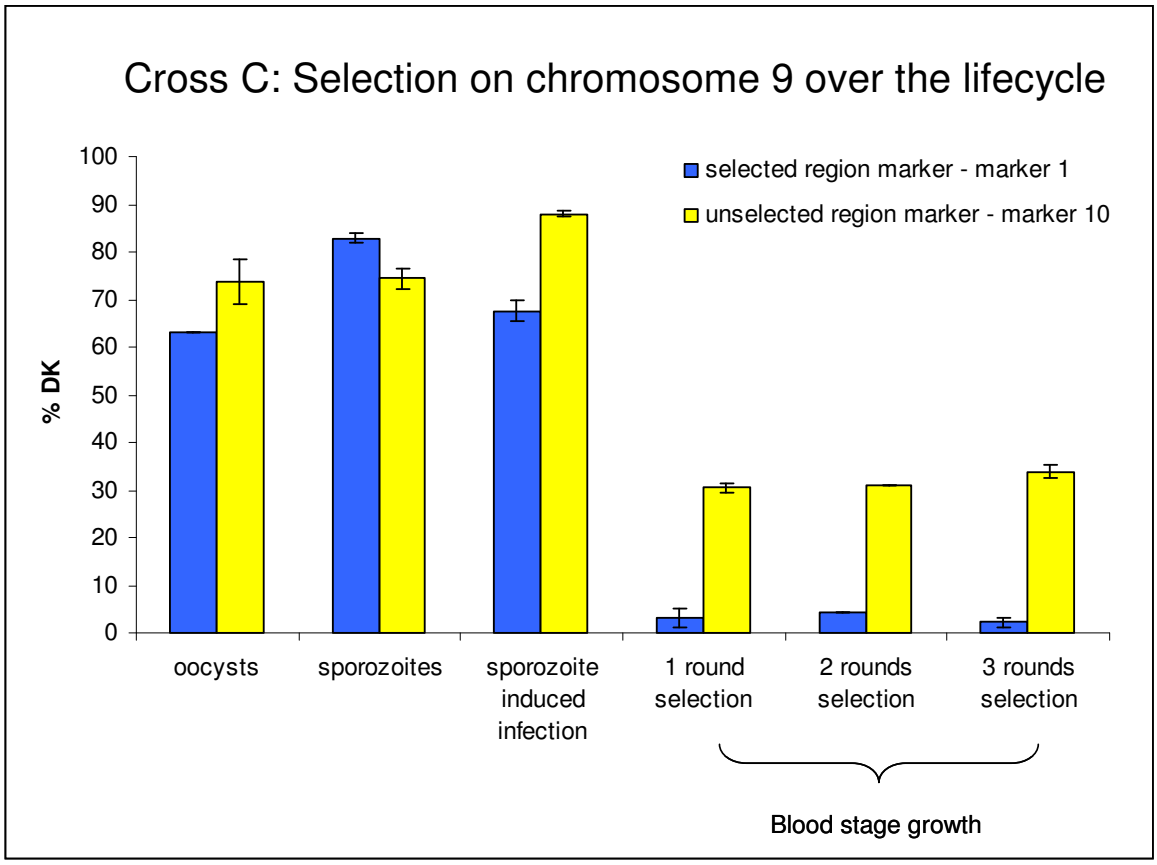
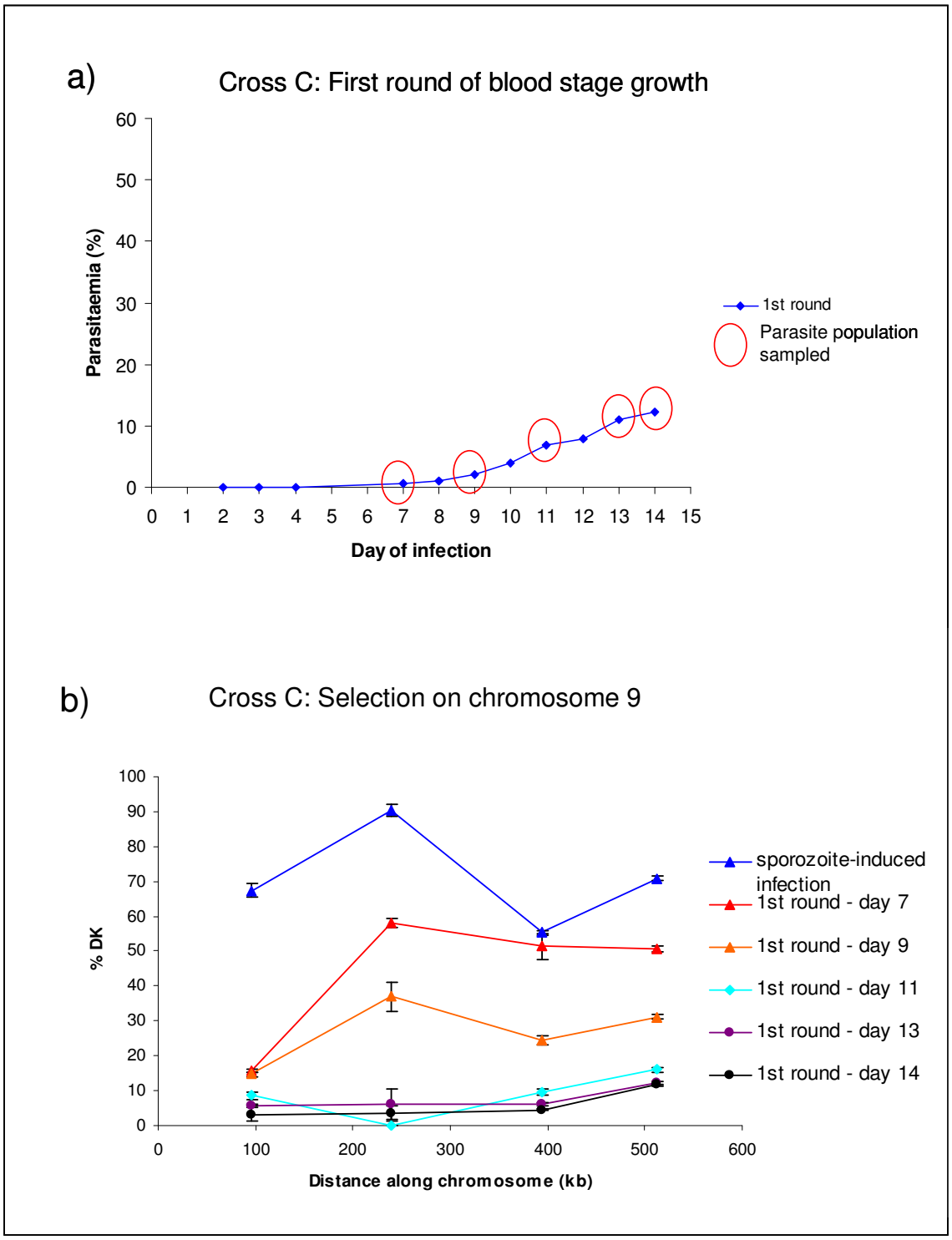


Figure 6.4
(a) Daily parasitaemia during the first round of cross C progeny blood stage growth selection, with days of parasite population sampling shown circled in red. (b) The proportion of cross C progeny inheriting DK at 4 marker positions on chromosome 9 (markers 1, 2, 3 and 4, see Appendix 1) in the sporozoite-induced infection and at 5 time-points taken during the 1st round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.

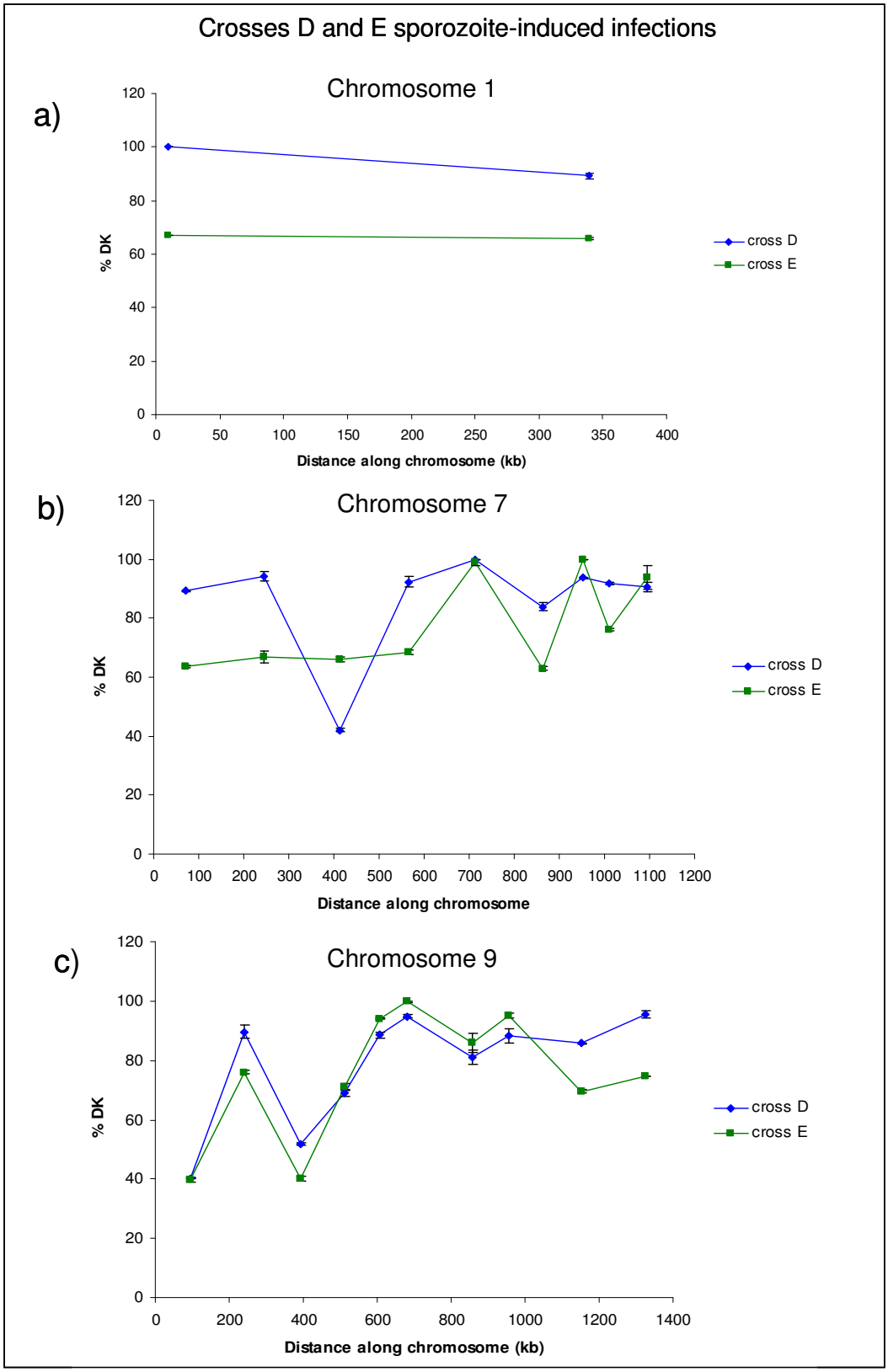


unselected (sporozoite-induced infection) level of DK in this region was around 70-80% (Figure 6.4b). After 7 days of blood stage growth (to 0.7% parasitaemia) after passage from this sporozoite-induced infection, DK marker 1 was reduced to around 15% whereas the other three DK markers remained around 50%, this difference was statistically significant (non-parametric Mann Whitney *U*-test: marker 1 v. marker 2 $p<0.01$, marker 1 v. marker 3 $p=0.02$, and marker 1 v. marker 4 $p=0.01$). By day 13, DK markers 1 and 3 were almost down to zero, with marker 4 still significantly less reduced at 10% (non-parametric Mann Whitney *U*-test: marker 1 v. marker 4 $p=0.02$, and marker 3 v. marker 4 $p=0.01$). Although marker 2 was also reduced close to zero, due to a larger spread of data, it was not significantly less than marker 4 ($p=0.31$).

6.3.4. Analysis of DK Marker Inheritance in Cross D and E Sporozoite-Induced Infections

Material from sporozoite-induced infections of crosses D and E was also collected and tested for DK marker inheritance across chromosomes 7 and 9, along with the unaffected chromosome 1. These sporozoite-induced infections were grown until day 4 at 1.93% parasitaemia for cross D and until day 3 at 0.58% parasitaemia for cross E. At this point, blood samples were taken and DK marker inheritance assessed (Figure 6.5). Cross D was almost 100% DK on chromosome 1, with cross E around 70% DK (Figure 6.5a). On chromosome 7, marker 3 was reduced in cross D from a background level of around 90% to 40%; however, markers 5, 7 and 9 in cross E were actually higher than the background level of around 60% DK (Figure 6.5b). Across chromosome 9, only DK markers 1 and 3 showed signs of significant reduction from background levels, to between 40-50% in both crosses (Figure 6.5c).

Figure 6.5.
The proportion of the cross D and E progeny inheriting DK at (a) 2 marker positions on chromosome 1, (b) 9 marker positions on chromosome 7 and (c) 10 marker positions on chromosome 9, after the sporozoite-induced infections had emerged in the blood and before passage on for the 1st round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



6.4. Discussion

From the original cross A and B progeny, and backcross 1, 2 and 3 progeny, it was clear that the pattern of marker inheritance across chromosomes 7 and 9 was already set by the first round of growth in the blood, and changed little over the subsequent rounds of growth. When DK markers did decrease over time it was most likely from a loss of residual DK parent, rather than a loss of particular recombinants, because all the DK markers decreased by the same amount. From the analysis of cross C DNA extracted from oocysts, sporozoites and the first parasites visible in the blood after a sporozoite-induced infection, it was evident that selection on the region of interest on chromosome 9 had not yet taken place and had only occurred, therefore, during the first round of blood stage growth. This ruled out the possibility that selection on the cross progeny pool at either the mosquito or liver stage of the life-cycle, was responsible for producing the dramatic loss of DK marker inheritance on chromosome 9.

It was then important to determine when, during the first round of growth in the blood, selection on the left-hand region of chromosome 9 in Cross C progeny was occurring. Cross C grew very slowly for this first round, only reaching 12% parasitaemia by day 14, but this length of time was sufficient to produce the strong selection on DK chromosome 9 (markers 1, 2 and 3 almost reduced to zero). However, on closer inspection of this 14-day period of growth, it was apparent that by day 7, when the parasitaemia was only 0.7%, DK marker 1 was already significantly more reduced than were DK markers 2, 3 and 4. Therefore, it is possible that in the cross C progeny, a DK gene in the region of marker 1 was selected against

first, at least as early as after 7 days of growth in the blood, and this was followed later by a DK gene in the region of markers 2 and 3. Due to the low parasitaemia (and thus low level of parasite DNA) of cross C before day 7 of the first round of selection, marker inheritance could not be assessed any earlier than this.

With the creation of crosses D and E, more information regarding the timing of selection could be gained. The cross progeny obtained from sporozoite-induced infections were grown for 4 days to 1.93% parasitaemia for cross D and for 3 days to 0.58% parasitaemia for cross E. Unlike in sporozoite-induced infections of cross C, a reduction in chromosome 9 DK marker 1 inheritance was already starting to be seen in sporozoite-induced infections of crosses D and E (c.f. Figure 6.3 and Figure 6.5c). It is unclear why selection was already beginning to occur on chromosome 9 after such a short period post-inoculation of sporozoites for crosses D and E, when it had not occurred in cross C over a similar time period. The time taken from inoculation of sporozoites to hepatic schizont rupture and release of merozoites into the blood in *P. chabaudi* is around 2 days. Therefore, in these two crosses, after 1 or 2 days of the intra-erythrocytic cycle (1 to 2 cycles of 24 hours), recombinants with DK alleles in this region of chromosome 9 were already starting to be lost from the parasite pool. However, a complete loss of these recombinants would be expected to take several more cycles of blood stage growth, as observed for cross C.

The inheritance of DS alleles on the left hand side of chromosome 9 by cross C, D and E progeny did not immediately mean that the populations of parasites were fast-growing. It was only after passage into a new host when they became fast-growing,

reaching 40-50% parasitaemia by days 8-9 of the second round of selection, which was a rate of growth comparable to pure DS (see Chapter 5, Figure 5.2). Therefore, although the genotypes of these recombinant pools were established early on by growth selection, it was only when they were passaged into a new host that the fast growth phenotypes of these pools emerged.

This could be explained if the presence of a large number of slow-growing recombinants and DK parentals affected the initial growth of otherwise fast-growing recombinants and DS parentals (as seen for DK affecting DS growth in Chapter 3). If this was the case, total parasitaemias might be lower than if fast-growing recombinants and DS parasites were in the majority in the starting inoculum. Even given the presence of a large number of slow growing parasites, fast-growing recombinants should increase in numbers over time. However, their growth might be limited by an immune response developing to the prolonged infection (crosses C, D and E grew for at least 13 days in the host during their first round of selection). On sub-inoculation into a new host, these fast-growing recombinants would be released from the previous heavy competition with slow-growing parasites and from the pressure of the immune response. This change of environment might lead to the full expression of the fast-growing, DS phenotype.

From these studies on the extent, location and timing of DK marker reduction, we can now conclude that a gene or genes in the left-hand region of *P. chabaudi* chromosome 9 is a major determinant of the blood stage growth, and thus

pathogenicity, of *P. c. adami*. Further loci on chromosomes 6 and 7 are also probably involved, to lesser and more complex extent.

Several genetic studies have been carried out in *P. chabaudi, P. yoelii* and *P. falciparum* in which growth phenotypes have been associated with different genomic regions. However, none of these genes or genomic regions correspond to those identified in the present LGS analysis. In the original classical genetics study using cloned progeny from a cross between the fast-growing, pathogenic *P. yoelii* YM strain and the slow-growing, non-pathogenic *P. yoelii* AC strain, the lethal growth phenotype of YM was inherited as a Mendelian trait (Walliker et al. 1976). A recent LGS analysis in this laboratory using the related *P. yoelii* 33X and YM has confirmed that the growth difference is due to a single locus, a region of chromosome 13 (Pattaradilokrat 2008). However, this region also corresponds to one on chromosome 13 in *P. chabaudi* which was not identified in the present LGS analysis. Given that there is a visible switch to a greatly enhanced normocyte invasion rate in *P. yoelii* YM, which corresponds to the rapid increase in parasitaemia, and that a similar event is not observed in *P. c adami* DS, it is perhaps unlikely that these two parasite strains should share the same genetic factors which control their growth.

A specific genetic linkage analysis of differences in growth phenotypes in *P. chabaudi* has not previously been carried out. However, from other *P. chabaudi* genetic studies designed to look at the inheritance of drug resistance genes, there was some anecdotal evidence that particular regions of the genome might be involved in

growth. Three markers on *P. chabaudi* chromosome 11 and 1 marker on chromosome 14 were weakly associated with growth between days 4 and 6 of infection, in a linkage analysis of *P. chabaudi* AJ x AS (3CQ) crosses (Carlton et al. 1998a). The *P. chabaudi* AJ strain is slightly faster growing than the chloroquine resistant AS(3CQ) strain and although cross progeny were analysed for marker linkage to the chloroquine resistant phenotype, it was also apparent that in the control group of non-drug-treated mice, faster growth between days 4-6 was associated with these four markers. Similarly, in a linkage analysis of *P. chabaudi* AJ x AS(50S/P) crosses to discover the genetic loci involved in sulphadoxine / pyrimethamine resistance, loci on chromosome 5 and 13 were thought to possibly confer the growth advantage of AJ over AS(50S/P) (Hayton et al. 2002). Again, these loci are all different to those on chromosomes 6, 7 and 9 as identified by the present LGS analysis. This indicates that either the linkage of these markers to the AJ growth phenotype was not significant, or that separate mechanisms exist for the control of growth in the two subspecies *P. c. adami* and *P. c. chabaudi.*

The genetic basis of the difference in multiplication rate between *P. falciparum* isolates is now starting to be addressed. The *hrp3* gene from *P. falciparum* 3D7 was found to mark a linkage group whose inheritance was strongly favoured in the cloned cross progeny of a *P. falciparum* 3D7 x HB3 cross (Wellems et al. 1987). These cross progeny had *in vitro* multiplication rates comparable to the 3D7 parent and were therefore, much faster growing than the HB3 parent. The *hrp3* gene is near to one end of chromosome 13 of 3D7 but has apparently been deleted in HB3, and this genomic region might be involved in determining the faster growth phenotype of

3D7. However, this region corresponds to chromosome 11 in *P. chabaudi* and *hrp3* itself does not have an orthologue in *P. chabaudi*.

There are plans for a quantitative trait locus (QTL) analysis of the difference in multiplication rate between the *P. falciparum* Dd2 and HB3 strains (Reilly et al. 2007), using the cloned cross progeny from a Dd2 x HB3 cross (Wellems et al. 1990). The Dd2 strain grows faster *in vitro* than the HB3 strain and this may be due to a combination of the following differences: Dd2 completes its intra-erythrocytic cell cycle an average of 6 hours faster than HB3 and this is due to a more rapid progression of rings to the trophozoite stage, Dd2 produces approximately 2 more merozoites per schizont than HB3 and invades approximately 3 times as many erythrocytes than Dd2 (Reilly et al. 2007). There are also differences in drug susceptibility between the two strains. Therefore it is clear that many factors and therefore, many parasite genes, might contribute to the difference in multiplication rate between HB3 and Dd2.

Unfortunately, it is not possible to measure many of these parameters in *P. c. adami* DS and DK, since there is no robust *in vitro* culture system available. However, it will be very interesting to discover if any of the genes identified by the QTL analysis of HB3 and Dd2 have orthologues in *P. chabaudi* in the regions identified by the present LGS analysis. The main caveat is that *P. falciparum* studies are carried out *in vitro* and therefore, any *P. falciparum* genes which may be involved in host-parasite interactions determining parasite growth will not be identified.

This raises an important point about the results of the present LGS analysis. As well as parasite genes that might be involved in metabolism and invasion, parasite genes which lead to host-parasite interactions, and that might influence the control of parasite proliferation in the host, must also be considered. These parasite genes might be ones that encode antigens, for example. It is possible that the presence of a particularly immunogenic antigen in a parasite strain such as DK could lead to a strong immune response against it, and thus, lead to lower parasite growth in the host. If a parasite strain such as DS was able to evade an inhibitory immune response against it, it might reach a higher parasitaemia in the host. However, the exact role of the host immune response in determining the growth characteristics of malaria parasites is difficult to determine because any scenario, from a total involvement of the host immune response to none at all, might depend on the particular hosts and parasites involved.

Parasite genes which might be involved in host-parasite interactions leading to the control of growth should also be identified by the present LGS analysis because, unlike genetic studies in *P. falciparum*, infections are carried out *in vivo*. Therefore, the presence of an asparagine-rich antigen in the region of chromosome 9 under selection is especially interesting. So also is the subtelomeric region of this chromosome, which must be expected to contain many variant gene family members which might have a role in antigenic variation and therefore, in escape from the immune response (Fischer et al. 2003).

A further point to note is that the difference in DS and DK growth characteristics is only apparent on day 6 of infection and onwards, and this timing could coincide with the activation of the adaptive immune response. In *P. c. adami* DK at least, B-cell deficient mice were able to control the first peak of parasitaemia, but in T-cell deficient mice, the parasitaemia was not reduced and mice died 7-9 weeks post infection (Grun and Weidanz 1981). However, the removal of T cells did not mean that the DK infection resembled that of the DS infection in which mice die after only 9 days of infection from much higher parasitaemias. Therefore, although the control of the first peak of parasitaemia in *P. c. adami* may be T-cell dependent, a complete evasion of this protective response by DS would not solely account for the rapid lethality and high parasitaemia of this strain.

Although cell cycle time and number of merozoites per schizont cannot be compared between DS and DK, it was clear from Chapter 2 that DK was more restricted in its invasion of erythrocytes than DS. Therefore, it is perhaps most likely that a combination of intrinsic parasite multiplication ability and particular host-parasite interactions leads to the differential growth characteristics of DS and DK. As only one mouse host; inbred laboratory female CBA/Ca mice, was used here, it would be important to repeat the LGS analysis using DS and DK cross progeny selected in at least one other strain, and perhaps gender, of mouse. This might lead to the identification of other genetic loci involved in growth determination and therefore, might indicate which loci, if any, from the present LGS analysis in CBA mice are involved in host-parasite interactions influencing growth characteristics.

Chapter 7: Discussion

7.1. Genetic Analysis of Growth Characteristics using LGS

Linkage group selection (LGS) has been developed for the rapid and economical identification of genetic loci which determine interesting phenotypes of rodent malaria parasites (Carter et al. 2007). Previous, published LGS analyses have investigated the genetic basis of drug resistance and strain specific protective immunity in *P. chabaudi* (Culleton et al. 2005; Martinelli et al. 2005b; Hunt et al. 2007; Pattaradilokrat et al. 2007). More recently, an LGS analysis of a different phenotype, growth, has been carried out in this laboratory using two strains of *P. yoelii*, 33X and YM (Pattaradilokrat 2008). The lethal, rapid growth property of YM arose during maintenance in the laboratory, and this strain does not have the strong preference for reticulocyte invasion which is typical of the other, slow-growing and non-lethal, *P. yoelii* strains. In contrast to the *P. yoelii* species, in which there is only one fast-growing strain (Pattaradilokrat et al. 2008), there are quite large differences in the growth properties of a number of *P. chabaudi* strains. These differences appear to have been stable over time and there is no obvious difference in erythrocyte invasion preference. Therefore, it is interesting to also investigate the growth differences between *P. chabaudi* strains using the LGS approach.

In *P. chabaudi* infections of laboratory mice, strains which reach higher parasitaemias have been found to cause more weight loss, anaemia and death, than those which reach lower parasitaemias (Mackinnon and Read 1999; Mackinnon and Read 2004). As these distinct parasite growth properties are associated with the

pathogenicity of infection, determining the genetic basis of the differences in growth should also lead to the identification of parasite virulence factors. Genetic analyses of important phenotypes, such as growth, in rodent malaria parasites, have the power to inform our knowledge about the related human malaria parasite *P. falciparum*, and have ethical and financial advantages. There is much we do not yet understand about how the same species of malaria parasite causes infections of diverse clinical severity. Heterogeneity in the host population must be a factor, but in spite of this, it is clear from the historical use of induced malaria infections to treat neurosyphilis patients, that individual strains of *P. falciparum* were phenotypically different (James et al. 1932; Covell and Nicol 1951). It is also apparent today, in particular geographical regions, that naturally occurring isolates of *P. falciparum* can have distinct growth and invasion characteristics which may be linked to their pathogenicity (Chotivanich et al. 2000; Dondorp et al. 2005).

LGS was employed here to investigate the genetic basis for the different growth properties of two strains DS and DK, of *P. c. adami*, a subspecies of *P. chabaudi* (Carter and Walliker 1976). DS and DK, in particular, were chosen due to the very large difference in growth between them, and the association of this with pathogenicity. In the experience of this laboratory, DS is a fast-growing, pathogenic strain and DK is a slow-growing, non-pathogenic strain. These characteristics were apparent in the original thicket rat isolates from Congo (Brazzaville) in the 1970s (Carter and Walliker 1976) and have not changed over time.

7.2. Findings from Chapters 2 and 3

Growth characteristics, and other properties relevant to LGS analysis, were examined in single and mixed infections of DS and DK, as detailed in Chapters 2 and 3. Groups of laboratory mice were inoculated with either DS or DK, and the resulting blood infections were monitored for several days. Daily parasitaemia counts demonstrated the clear difference in growth between the two strains; DS reached a very high parasitaemia, 60%, after 9 days of growth in the blood, whereas DK only reached a maximum of around 15% parasitaemia over the same number of days. The high parasitaemia of DS led to significantly greater anaemia and weight loss than DK, and mice infected with DS had to be euthanased by day 9. In contrast, DK infections were of very low pathogenicity.

It was also apparent from examination of blood smears, that in DK infections, multiply-infected red blood cells were found twice as frequently as expected. This indicated that DK invasion was restricted to a sub-population of red blood cells. This was not the case in DS infections, where the number of multiply-infected red blood cells was the same as that expected from a random process of red blood cell invasion. As a preference for reticulocyte invasion was not observed in DK infections, this could not explain the selectivity of invasion. However, as the degree of DK invasion selectivity did not change over time, it was unlikely that it could account for the difference in parasitaemia between DS and DK, which started to be apparent after day 5 of infection.

Two additional characteristics of these strains were investigated, because of the requirement for growth in mixed infections and transmission to mosquitoes, in LGS analysis. Firstly, in infections of two different laboratory mouse strains, the more pathogenic DS was significantly less successful at transmission to mosquitoes than the less pathogenic DK, over the course of infection. Secondly, when DS and DK were grown together in mixed infections, their growth properties changed depending on the proportion of each present in the initial inoculum; under particular conditions, the slow-growing DK was able to reduce the growth of DS, and vice versa. LGS requires the production of a large number of recombinants, and for this, optimal crossing conditions are required, where equal proportions of each parent are present in the mosquito. Therefore, the investigations of Chapters 2 and 3 provided useful information about the different growth and transmission behaviours of the two strains. In particular, they indicated that increasing the amount of DS relative to DK in a mixed infection, in order to improve the chances of equal transmission of both strains for a cross, needed to be balanced against the prospect of DS suppressing the growth and perhaps, transmission, of DK. As well as having implications for the LGS analysis, the results of Chapters 2 and 3 were also interesting in the context of the literature on transmission and mixed-strain infections of *P. c. chabaudi*.

7.3. Findings from Chapter 4

Two crosses were created between DS and DK, and over three rounds of growth in the blood, the pooled cross progeny were selected for the fast growth characteristics (high parasitaemia) of the DS parental strain. AFLP marker inheritance patterns indicated that there was a low proportion of DK in the crosses relative to DS, and

that many DK markers were strongly reduced by growth selection. These crosses were then backcrossed to DK, creating three backcrosses which were also selected for fast growth, and the same AFLP markers were examined. Backcrossing had the desired effect of increasing the background levels of DK, resulting in a larger range of DK marker inheritance values. Ten DK markers were reduced in inheritance in all three backcrosses and these were also found to be strongly reduced in both crosses. Five of these markers could be located in the *P. chabaudi* genome but the remaining five could not, due to difficulties in obtaining marker sequences or in matching marker sequences to the *P. falciparum* database (which is a required step for locating a marker in the non-annotated *P. chabaudi* genome).

Of the five markers which could be located in *P. chabaudi*, three were distributed across chromosome 7, one was located on chromosome 6 and one on chromosome 9. This showed that the growth difference between DS and DK was not determined by a single genetic locus. In the only other growth-phenotype LGS analysis that has been carried out to-date, i.e. that of two strains of *P. yoelii*, 33X and YM (Pattaradilokrat 2008), a single locus was identified as the determinant of the growth difference, a region of chromosome 13. This, clearly, was not the case in *P. c. adami*, as at least 3 chromosomes appeared to be involved. Of the ten DK markers of interest, the two which were most reduced were located on chromosomes 7 and 9. Therefore, these two chromosomes were investigated further in Chapter 5. The potential involvement of a locus or loci on chromosome 6 was not explored in this thesis, in order to focus efforts on the two chromosomes which appeared to be most important.

7.4. Findings from Chapter 5

SNP markers specific to either parent were generated at regular intervals across chromosomes 7 and 9. The inheritance of these DK markers was quantified by pyrosequencing, in the fully growth-selected cross and backcross progeny from Chapter 4, and in three new cross progeny pools that had been created and then selected for fast growth. Comparing all crosses and backcrosses, DK marker inheritance across chromosome 7 was reduced to varying degrees by growth selection, but there was no region in particular which was consistently very strongly selected against. This might indicate that several loci, which are relatively minor growth determinants, are present across chromosome 7.

In contrast, on chromosome 9, only those DK markers on the left-hand side of the chromosome were consistently and strongly reduced by growth selection. This region, comprising around 400kb, is expected to contain a gene (or genes) with a major effect on the control of growth in *P. c. adami*. There are at least 89 genes in this region, many with no function assigned, and therefore it is difficult to speculate which might be potential candidates for growth determination. Genes involved in metabolism, in red blood cell invasion and in interaction with the host immune system, for example, might all influence the growth properties of a malaria parasite. However, none of the genes in this region of chromosome 9 have been specifically associated with the determination of growth in other studies.

Given that the region of interest on chromosome 9 is very large, one explanation might be that it contains several closely-linked genes which are important for growth,

raising the possibility that functionally-linked genes might be physically-linked in *Plasmodium* (Carlton 1999). Physical clustering of genes which are expressed at the same stage of the erythrocytic cycle has been demonstrated in *P. falciparum* (Florens et al. 2002). However, an analysis of the functionally annotated genes in the region of interest on chromosome 9 did not show any clustering of genes with similar biological function. Another possibility is that there are variations in recombination rate across the genome of *P. chabaudi*, as has been demonstrated for one chromosome in *P. falciparum* (Mu et al. 2005). Therefore, if recombination is reduced in this region of chromosome 9, i.e. if there is a "cold-spot", then there will be a greater degree of linkage disequilibrium between markers in this region than expected.

7.5. Findings from Chapter 6

It was clear from the original crosses and backcrosses that the DK marker inheritance patterns across chromosomes 7 and 9 changed little over the three rounds of blood passage for fast growth selection. However, selection on the region of interest on chromosome 9 in particular, was not found to occur in the cross progeny pool at either the mosquito or liver stage of the life-cycle. Instead, it had occurred by the end of a single round of passage in the blood, with the loss of recombinants possessing DK alleles on chromosome 9 beginning to occur after 1 or 2 days of blood stage growth. Therefore, the region of chromosome 9 identified by LGS was important for growth in the blood, and not, in fact, in any other stage of the lifecycle.

In conclusion, it is evident that a gene or genes in the left-hand region of *P. chabaudi* chromosome 9 is a major determinant of the blood stage growth properties, and thus pathogenicity, of *P. c adami*. Further loci on chromosomes 6 and 7 are also probably involved, albeit to lesser and more complex extent. The genetic loci identified in this study have not been linked to growth determination in the small number of other studies that have been carried out in *P. chabaudi, P. yoelii* and *P. falciparum*. Therefore, there may be several different mechanisms by which the growth properties of malaria parasites are determined. Narrowing down the locus of interest on chromosome 9 in particular, to identify the gene or genes that have the major effect on growth in these parasites, is now a priority.

7.6. Future Work

By cloning-out fast-growing recombinant cross progeny and measuring DK marker inheritance at several positions in the region of interest on chromosome 9, it should be possible to narrow this region down or to confirm if the whole region is linked to fast growth. Cloning-out is a strategy that has been partially explored, with limited success, in experiments described in Appendix II. The cloning of recombinant cross progeny is a partial return to the classical genetics approach, although by using LGS first, the areas of the genome which would need to be investigated for linkage have been greatly reduced. Once a region has been narrowed down sufficiently, it should be possible to sequence the respective regions in the DS and DK genomes and compare them for intragenic non-synonymous differences. However, it might be that a genetic difference in a regulatory region, leading to a difference in gene expression, is the key determinant of the growth difference. In this case, a comparison between

strains, of the differences in expression of genes in the region of interest, could be made by using RT-PCR or by microarray, if available in the future. Furthermore, the development of massively parallel sequencing technology will make it possible in the near future to fully sequence many thousands of pooled recombinant progeny, and its use in the context of LGS should be explored.

The ultimate test of the association of a particular gene with a particular phenotype, is to transfect the gene from parasite strain A into strain B, and to observe a change in phenotype from B to A. The genetic manipulation of *Plasmodium* still remains difficult, although new technologies are being developed (Balu and Adams 2007). The transfection efficiency of *P. falciparum* is very low, although that of the rodent malaria parasite *P. berghei* is significantly better. Due to the inability of *P. chabaudi* to grow *in vitro*, a successful genetic manipulation of this parasite species has not yet been reported. Therefore, should a candidate gene for growth determination in *P. c. adami* be identified, it is unlikely that it could be tested in the near future, through the transfection of DS with the DK version of the gene, and vice versa.

One thing that is clear from the work of this thesis is the consistency of the selection against the region of DK chromosome 9, when measured across several independent genetic crosses, selected for fast growth at different times. This has been carried out in the same CBA strain of inbred laboratory mice. It is possible that by carrying out growth selection in a different host strain, different parasite genetic loci to those found by the present study, might be under selection. This would be the case if the host immune response influences, at least partly, the growth characteristics of DS

and DK, and the host immune response to these parasites varies between strains of laboratory mice. It is evident from experiments in Chapter 2, that in C57 mice at least, the growth characteristics of these parasites are very similar to those in CBA mice. Therefore, another host strain, in which this is not the case, might be used to select the same cross and backcross progeny pools for fast growth, and the results compared to those obtained in the present study.

7.7. Summary

1. The general biological characteristics of *P. c. adami* DS and DK strains are as follows: DS is a fast-growing parasite strain which causes a pathogenic infection in laboratory mice whereas DK is a slow-growing parasite strain causing a non-pathogenic infection, DS produces fewer gametocytes and is a poorer transmitter to mosquitoes than DK, DK selectively invades a sub-population of red blood cells whereas DS invasion is unrestricted, and finally, both DS and DK can influence each other's growth in mixed infections.
2. An LGS analysis of the difference in growth between DS and DK was carried out, by analysing AFLP and SNP-marker inheritance in several cross and backcross progeny pools selected for fast growth.
3. Genetic loci on *P. chabaudi* chromosomes 6, 7 and 9 were shown to be linked to the determination of the growth characteristics of DS and DK.
4. Selection on genetic loci occurred only during growth of the parasites in the blood, and not during the mosquito or liver stage of the parasite life-cycle.

5. A region of chromosome 9, in particular, was very strongly linked to the control of growth in the blood and therefore, is expected to contain a gene (or genes) that have a major effect on parasite growth.

8. Bibliography

Ahmadian A, Ehn M, Hober S (2006) Pyrosequencing: history, biochemistry and future. Clin Chim Acta 363(1-2): 83-94.

Amino R, Thiberge S, Martin B, Celli S, Shorte S et al. (2006) Quantitative imaging of Plasmodium transmission from mosquito to mammal. Nat Med 12(2): 220-224.

Anders RF, Crewther PE, Edwards S, Margetts M, Matthew ML et al. (1998) Immunisation with recombinant AMA-1 protects mice against infection with Plasmodium chabaudi. Vaccine 16(2-3): 240-247.

Antia R, Yates A, de Roode JC (2008) The dynamics of acute malaria infections. I. Effect of the parasite's red blood cell preference. Proc Biol Sci 275(1641): 1449-1458.

Ariey F, Hommel D, Le Scanf C, Duchemin JB, Peneau C et al. (2001) Association of severe malaria with a specific Plasmodium falciparum genotype in French Guiana. J Infect Dis 184(2): 237-241.

Baer K, Klotz C, Kappe SH, Schnieder T, Frevert U (2007) Release of Hepatic Plasmodium yoelii Merozoites into the Pulmonary Microvasculature. PLoS Pathog 3(11): e171.

Balu B, Adams JH (2007) Advancements in transfection technologies for Plasmodium. Int J Parasitol 37(1): 1-10.

Barnwell JW, Galinski MR (1998) Chapter 7: Invasion of vertebrate cells : Erythrocytes, in Malaria: Parasite biology, pathogenesis and protection; Sherman IW, editor. Washington, DC: ASM Press.

Bell AS, de Roode JC, Sim D, Read AF (2006) Within-host competition in genetically diverse malaria infections: parasite virulence and competitive success. Evolution Int J Org Evolution 60(7): 1358-1371.

Blake DP, Shirley MW, Smith AL (2006) Genetic identification of antigens protective against coccidia. Parasite Immunol 28(7): 305-314.

Blake DP, Hesketh P, Archer A, Carroll F, Smith AL et al. (2004) Parasite genetics and the immune host: recombination between antigenic types of Eimeria maxima as an entree to the identification of protective antigens. Mol Biochem Parasitol 138(1): 143-152.

Boyd M, Stratman-Thomas W, Kitchen S (1936) On aquired immunity to Plasmodium falciparum. Am J Trop Med Hyg 16: 139-145.

Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J et al. (2003) The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol 1(1): E5.

Brewster DR, Kwiatkowski D, White NJ (1990) Neurological sequelae of cerebral malaria in children. Lancet 336(8722): 1039-1043.

Bruce MC, Alano P, Duthie S, Carter R (1990) Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. Parasitology 100 Pt 2: 191-200.

Bruce MC, Donnelly CA, Alpers MP, Galinski MR, Barnwell JW et al. (2000) Cross-species interactions between malaria parasites in humans. Science 287(5454): 845-848.

Carlton J, Mackinnon M, Walliker D (1998a) A chloroquine resistance locus in the rodent malaria parasite Plasmodium chabaudi. Mol Biochem Parasitol 93(1): 57-72.
Carlton JM (1999) Gene synteny across Plasmodium spp: could 'operon-like' structures exist? Parasitol Today 15(5): 178-179.
Carlton JM, Vinkenoog R, Waters AP, Walliker D (1998b) Gene synteny in species of Plasmodium. Mol Biochem Parasitol 93(2): 285-294.
Carlton JM, Hayton K, Cravo PV, Walliker D (2001) Of mice and malaria mutants: unravelling the genetics of drug resistance using rodent malaria models. Trends Parasitol 17(5): 236-242.
Carlton JM, Angiuoli SV, Suh BB, Kooij TW, Pertea M et al. (2002) Genome sequence and comparative analysis of the model rodent malaria parasite Plasmodium yoelii yoelii. Nature 419(6906): 512-519.
Carter R (1978) Studies on enzyme variation in the murine malaria parasites Plasmodium berghei, P. yoelii, P. vinckei and P. chabaudi by starch gel electrophoresis. Parasitology 76(3): 241-267.
Carter R, Walliker D (1975) New observations on the malaria parasites of rodents of the Central African Republic - Plasmodium vinckei petteri subsp. nov. and Plasmodium chabaudi Landau, 1965. Ann Trop Med Parasitol 69(2): 187-196.
Carter R, Walliker D (1976) Malaria parasites of rodents of the Congo (Brazzaville): Plasmodium chabaudi adami subsp. nov. and Plasmodium vinckei lentum Landau, Michel, Adam and Boulard, 1970. Ann Parasitol Hum Comp 51(6): 637-646.
Carter R, Gwadz R (1980) Infectiousness and gamete immunization in malaria. Research in Malaria JP Kreier (ed), Academic Press Inc Vol. 3: pp.263-298.
Carter R, Graves P (1988) "Gametocytes" Chapter 7 in Malaria, Principles and Practice of Malariology. WH Wernsdorfer and I McGregor (eds), Churchill Livingstone: pp 253-305.
Carter R, Mendis KN (2002) Evolutionary and historical aspects of the burden of malaria. Clin Microbiol Rev 15(4): 564-594.
Carter R, Mendis K (2006) Measuring malaria. Am J Trop Med Hyg 74(2): 187-188.
Carter R, Hunt P, Cheesman S (2007) Linkage Group Selection - A fast approach to the genetic analysis of malaria parasites. Int J Parasitol 37(3-4): 285-293.
Cheesman S, Creasey A, Degnan K, Kooij T, Afonso A et al. (2007) Validation of Pyrosequencing for accurate and high throughput estimation of allele frequencies in malaria parasites. Mol Biochem Parasitol 152(2): 213-219.
Chotivanich K, Udomsangpetch R, Simpson JA, Newton P, Pukrittayakamee S et al. (2000) Parasite multiplication potential and the severity of Falciparum malaria. J Infect Dis 181(3): 1206-1209.
Cogswell FB (1992) The hypnozoite and relapse in primate malaria. Clin Microbiol Rev 5(1): 26-35.
Conway DJ, Roper C, Oduola AM, Arnot DE, Kremsner PG et al. (1999) High recombination rate in natural populations of Plasmodium falciparum. Proc Natl Acad Sci U S A 96(8): 4506-4511.
Covell G, Nicol WD (1951) Clinical, chemotherapeutic and immunological studies on induced malaria. Br Med Bull 8(1): 51-55.

Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A et al. (2008) Plasmodium knowlesi malaria in humans is widely distributed and potentially life threatening. Clin Infect Dis 46(2): 165-171.
Cravo PV, Carlton JM, Hunt P, Bisoni L, Padua RA et al. (2003) Genetics of mefloquine resistance in the rodent malaria parasite Plasmodium chabaudi. Antimicrob Agents Chemother 47(2): 709-718.
Crewther PE, Matthew ML, Flegg RH, Anders RF (1996) Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strain-specific epitopes. Infect Immun 64(8): 3310-3317.
Culleton R, Martinelli A, Hunt P, Carter R (2005) Linkage group selection: rapid gene discovery in malaria parasites. Genome Res 15(1): 92-97.
Day KP, Karamalis F, Thompson J, Barnes DA, Peterson C et al. (1993) Genes necessary for expression of a virulence determinant and for transmission of Plasmodium falciparum are located on a 0.3-megabase region of chromosome 9. Proc Natl Acad Sci U S A 90(17): 8292-8296.
Deans AM, Lyke KE, Thera MA, Plowe CV, Kone A et al. (2006) Low multiplication rates of African Plasmodium falciparum isolates and lack of association of multiplication rate and red blood cell selectivity with malaria virulence. Am J Trop Med Hyg 74(4): 554-563.
Dearsly AL, Sinden RE, Self IA (1990) Sexual development in malarial parasites: gametocyte production, fertility and infectivity to the mosquito vector. Parasitology 100 Pt 3: 359-368.
DeRoode JC, Read AF, Chan BH, Mackinnon MJ (2003) Rodent malaria parasites suffer from the presence of conspecific clones in three-clone Plasmodium chabaudi infections. Parasitology 127(Pt 5): 411-418.
DeRoode JC, Helinski ME, Anwar MA, Read AF (2005a) Dynamics of multiple infection and within-host competition in genetically diverse malaria infections. Am Nat 166(5): 531-542.
DeRoode JC, Culleton R, Cheesman SJ, Carter R, Read AF (2004) Host heterogeneity is a determinant of competitive exclusion or co-existence in genetically diverse malaria infections. Proceedings of the Royal Society of London, Series B 271: 1073-1080.
DeRoode JC, Pansini R, Cheesman SJ, Helinski ME, Huijben S et al. (2005b) Virulence and competitive ability in genetically diverse malaria infections. Proc Natl Acad Sci U S A 102(21): 7624-7628.
Dondorp AM, Desakorn V, Pongtavornpinyo W, Sahassananda D, Silamut K et al. (2005) Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. PLoS Med 2(8): e204.
Dondorp AM, Lee SJ, Faiz MA, Mishra S, Price R et al. (2008) The Relationship between Age and the Manifestations of and Mortality Associated with Severe Malaria. Clin Infect Dis 47(2): 151-157.
Engelbrecht F, Felger I, Genton B, Alpers M, Beck HP (1995) Plasmodium falciparum: malaria morbidity is associated with specific merozoite surface antigen 2 genotypes. Exp Parasitol 81(1): 90-96.
Fahey JR, Spitalny GL (1987) Rapid parasite multiplication rate, rather than immunosuppression, causes the death of mice infected with virulent Plasmodium yoelii. Infect Immun 55(2): 490-493.

Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA et al. (2004) Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 52(4): 985-997.
Ferguson HM, Mackinnon MJ, Chan BH, Read AF (2003) Mosquito mortality and the evolution of malaria virulence. Evolution Int J Org Evolution 57(12): 2792-2804.
Ferreira MU, Nair S, Hyunh TV, Kawamoto F, Anderson TJ (2002) Microsatellite characterization of Plasmodium falciparum from cerebral and uncomplicated malaria patients in southern Vietnam. J Clin Microbiol 40(5): 1854-1857.
Field JW, Niven JC (1937) A note on prognosis in relation to parasite counts in acute subtertian malaria. Trans R Soc Trop Med Hyg 30(6): 569-574.
Fischer K, Chavchich M, Huestis R, Wilson DW, Kemp DJ et al. (2003) Ten families of variant genes encoded in subtelomeric regions of multiple chromosomes of Plasmodium chabaudi, a malaria species that undergoes antigenic variation in the laboratory mouse. Mol Microbiol 48(5): 1209-1223.
Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M et al. (2002) A proteomic view of the Plasmodium falciparum life cycle. Nature 419(6906): 520-526.
Freitas-Junior LH, Bottius E, Pirrit LA, Deitsch KW, Scheidig C et al. (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of P. falciparum. Nature 407(6807): 1018-1022.
Frevert U (2004) Sneaking in through the back entrance: the biology of malaria liver stages. Trends Parasitol 20(9): 417-424.
Gallup JL, Sachs JD (2001) The economic burden of malaria. Am J Trop Med Hyg 64(1-2 Suppl): 85-96.
Garcia JE, Puentes A, Patarroyo ME (2006) Developmental biology of sporozoite-host interactions in Plasmodium falciparum malaria: implications for vaccine design. Clin Microbiol Rev 19(4): 686-707.
Gardner MJ, Hall N, Fung E, White O, Berriman M et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419(6906): 498-511.
Garnham PCC (1966) Malaria parasites and other haemosporidia. Oxford: Blackwell Scientific Publications.
Gaur D, Mayer DC, Miller LH (2004) Parasite ligand-host receptor interactions during invasion of erythrocytes by Plasmodium merozoites. Int J Parasitol 34(13-14): 1413-1429.
Gilles HM (1993) Bruce-Chwatt's Essential Malariology, 3rd Edition, Chapter 2.
Grech K, Martinelli A, Pathirana S, Walliker D, Hunt P et al. (2002) Numerous, robust genetic markers for Plasmodium chabaudi by the method of amplified fragment length polymorphism. Mol Biochem Parasitol 123(2): 95-104.
Greenberg J (1956) Mixed lethal strains of Plasmodium gallinaceum: drug-sensitive, transferable (SP) x drug-resistant, non-transferable (BI). Exp Parasitol 5(4): 359-370.
Greenberg J, Trembley HL (1954a) The apparent transfer of pyrimethamine-resistance from the BI strain of Plasmodium gallinaceum to the M strain. J Parasitol 40(6): 667-672.
Greenberg J, Trembley HL (1954b) Infections produced by mixed strains of Plasmodium gallinaceum in chicks. J Parasitol 40(3): 336-340.

Grun JL, Weidanz WP (1981) Immunity to Plasmodium chabaudi adami in the B-cell-deficient mouse. Nature 290(5802): 143-145.
Gupta S, Hill AV, Kwiatkowski D, Greenwood AM, Greenwood BM et al. (1994) Parasite virulence and disease patterns in Plasmodium falciparum malaria. Proc Natl Acad Sci U S A 91(9): 3715-3719.
Hall N, Karras M, Raine JD, Carlton JM, Kooij TW et al. (2005) A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science 307(5706): 82-86.
Hastings IM, Paget-McNicol S, Saul A (2004) Can mutation and selection explain virulence in human P. falciparum infections? Malar J 3: 2.
Hayton K, Ranford-Cartwright LC, Walliker D (2002) Sulfadoxine-pyrimethamine resistance in the rodent malaria parasite Plasmodium chabaudi. Antimicrob Agents Chemother 46(8): 2482-2489.
Hernandez-Valladares M, Naessens J, Nagda S, Musoke AJ, Rihet P et al. (2004) Comparison of pathology in susceptible A/J and resistant C57BL/6J mice after infection with different sub-strains of Plasmodium chabaudi. Exp Parasitol 108(3-4): 134-141.
Holder AA, Freeman RR (1981) Immunization against blood-stage rodent malaria using purified parasite antigens. Nature 294(5839): 361-364.
Hunt P, Martinelli A, Fawcett R, Carlton J, Carter R et al. (2004) Gene synteny and chloroquine resistance in Plasmodium chabaudi. Mol Biochem Parasitol 136(2): 157-164.
Hunt P, Afonso A, Creasey A, Culleton R, Sidhu AB et al. (2007) Gene encoding a deubiquitinating enzyme is mutated in artesunate- and chloroquine-resistant rodent malaria parasites. Mol Microbiol 65(1): 27-40.
Hutagalung R, Wilairatana P, Looareesuwan S, Brittenham GM, Aikawa M et al. (1999) Influence of hemoglobin E trait on the severity of Falciparum malaria. J Infect Dis 179(1): 283-286.
James SP, Nichol WD, Shute PG (1932) A study of induced malignant tertian malaria. Proceedings of the Royal Society of Medicine 25: 1153-1186.
Janse CJ, Ramesar J, van den Berg FM, Mons B (1992) Plasmodium berghei: in vivo generation and selection of karyotype mutants and non-gametocyte producer mutants. Exp Parasitol 74(1): 1-10.
Janssen CS, Phillips RS, Turner CM, Barrett MP (2004) Plasmodium interspersed repeats: the major multigene superfamily of malaria parasites. Nucleic Acids Res 32(19): 5712-5720.
Janssen CS, Barrett MP, Lawson D, Quail MA, Harris D et al. (2001) Gene discovery in Plasmodium chabaudi by genome survey sequencing. Mol Biochem Parasitol 113(2): 251-260.
Jarra W, Brown KN (1989) Invasion of mature and immature erythrocytes of CBA/Ca mice by a cloned line of Plasmodium chabaudi chabaudi. Parasitology 99 Pt 2: 157-163.
Jeffery GM (1966) Epidemiological significance of repeated infections with homologous and heterologous strains and species of Plasmodium. Bull World Health Organ 35(6): 873-882.
Kaestli M, Cockburn IA, Cortes A, Baea K, Rowe JA et al. (2006) Virulence of malaria is associated with differential expression of Plasmodium falciparum var gene subgroups in a case-control study. J Infect Dis 193(11): 1567-1574.

Karunaweera ND, Carter R, Grau GE, Kwiatkowski D, Del Giudice G et al. (1992) Tumour necrosis factor-dependent parasite-killing effects during paroxysms in non-immune Plasmodium vivax malaria patients. Clin Exp Immunol 88(3): 499-505.
Killick-Kendrick R, Peters W (1978) Rodent Malaria. London: Academic Press.
Kooij TW, Carlton JM, Bidwell SL, Hall N, Ramesar J et al. (2005) A Plasmodium Whole-Genome Synteny Map: Indels and Synteny Breakpoints as Foci for Species-Specific Genes. PLoS Pathog 1(4): e44.
Kreuels B, Kobbe R, Adjei S, Kreuzberg C, von Reden C et al. (2008) Spatial Variation of Malaria Incidence in Young Children from a Geographically Homogeneous Area with High Endemicity. J Infect Dis 197(1): 85-93.
Kun JF, Schmidt-Ott RJ, Lehman LG, Lell B, Luckner D et al. (1998) Merozoite surface antigen 1 and 2 genotypes and rosetting of Plasmodium falciparum in severe and mild malaria in Lambarene, Gabon. Trans R Soc Trop Med Hyg 92(1): 110-114.
Kyriacou HM, Stone GN, Challis RJ, Raza A, Lyke KE et al. (2006) Differential var gene transcription in Plasmodium falciparum isolates from patients with cerebral malaria compared to hyperparasitaemia. Mol Biochem Parasitol 150(2): 211-218.
Lalloo DG, Shingadia D, Pasvol G, Chiodini PL, Whitty CJ et al. (2007) UK malaria treatment guidelines. J Infect 54(2): 111-121.
Landau I (1965) [Description of Plasmodium Chabaudi N. Sp., Parasite of African Rodents.]. C R Hebd Seances Acad Sci 260: 3758-3761.
Law PJ, Claudel-Renard C, Joubert F, Louw AI, Berger DK (2008) MADIBA: a web server toolkit for biological interpretation of Plasmodium and plant gene clusters. BMC Genomics 9: 105.
Le Scanf C, Vigan-Womas I, Contamin H, Guillotte M, Bischoff E et al. (2007) Rosetting is associated with increased Plasmodium falciparum in vivo multiplication rate in the Saimiri sciureus monkey. Microbes Infect 10(4): 447-451.
Mackinnon MJ, Read AF (1999) Genetic relationships between parasite virulence and transmission in the rodent malaria Plasmodium chabaudi. Evolution 53(3): 689-703.
Mackinnon MJ, Read AF (2003) The effects of host immunity on virulence-transmissibility relationships in the rodent malaria parasite Plasmodium chabaudi. Parasitology 126(Pt 2): 103-112.
Mackinnon MJ, Read AF (2004) Virulence in malaria: an evolutionary viewpoint. Philos Trans R Soc Lond B Biol Sci 359(1446): 965-986.
Mackinnon MJ, Walker PR, Rowe JA (2002) Plasmodium chabaudi: rosetting in a rodent malaria model. Exp Parasitol 101(2-3): 121-128.
Marsh K (1992) Malaria--a neglected disease? Parasitology 104 Suppl: S53-69.
Martha RC, Tada MS, Ferreira RG, Silva LH, Wunderlich G (2007) Microsatellite characterization of Plasmodium falciparum from symptomatic and non-symptomatic infections from the Western Amazon reveals the existence of non-symptomatic infection-associated genotypes. Mem Inst Oswaldo Cruz 102(3): 293-298.

Martinelli A, Hunt P, Cheesman SJ, Carter R (2004) Amplified fragment length polymorphism measures proportions of malaria parasites carrying specific alleles in complex genetic mixtures. Mol Biochem Parasitol 136(2): 117-122.
Martinelli A, Hunt P, Fawcett R, Cravo PV, Walliker D et al. (2005a) An AFLP-based genetic linkage map of Plasmodium chabaudi chabaudi. Malar J 4(1): 11.
Martinelli A, Cheesman S, Hunt P, Culleton R, Raza A et al. (2005b) A genetic approach to the de novo identification of targets of strain-specific immunity in malaria parasites. Proc Natl Acad Sci U S A 102(3): 814-819.
McRobert L, McConkey GA (2002) RNA interference (RNAi) inhibits growth of Plasmodium falciparum. Mol Biochem Parasitol 119(2): 273-278.
Miller LH, Baruch DI, Marsh K, Doumbo OK (2002) The pathogenic basis of malaria. Nature 415(6872): 673-679.
Miller RL, Ikram S, Armelagos GJ, Walker R, Harer WB et al. (1994) Diagnosis of Plasmodium falciparum infections in mummies using the rapid manual ParaSight-F test. Trans R Soc Trop Med Hyg 88(1): 31-32.
Min-Oo G, Gros P (2005) Erythrocyte variants and the nature of their malaria protective effect. Cell Microbiol 7(6): 753-763.
Mu J, Awadalla P, Duan J, McGee KM, Joy DA et al. (2005) Recombination hotspots and population structure in Plasmodium falciparum. PLoS Biol 3(10): e335.
Nachman MW (2002) Variation in recombination rate across the genome: evidence and implications. Curr Opin Genet Dev 12(6): 657-663.
Nair S, Williams JT, Brockman A, Paiphun L, Mayxay M et al. (2003) A selective sweep driven by pyrimethamine treatment in southeast asian malaria parasites. Mol Biol Evol 20(9): 1526-1536.
Naotunne TD, Rathnayake KD, Jayasinghe A, Carter R, Mendis KN (1990) Plasmodium cynomolgi: serum-mediated blocking and enhancement of infectivity to mosquitoes during infections in the natural host, Macaca sinica. Exp Parasitol 71(3): 305-313.
Naotunne TS, Karunaweera ND, Del Giudice G, Kularatne MU, Grau GE et al. (1991) Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J Exp Med 173(3): 523-529.
Nwakanma D, Kheir A, Sowa M, Dunyo S, Jawara M et al. (2008) High gametocyte complexity and mosquito infectivity of Plasmodium falciparum in the Gambia. Int J Parasitol 38(2): 219-227.
Pain A, Ferguson DJ, Kai O, Urban BC, Lowe B et al. (2001) Platelet-mediated clumping of Plasmodium falciparum-infected erythrocytes is a common adhesive phenotype and is associated with severe malaria. Proc Natl Acad Sci U S A 98(4): 1805-1810.
Pattaradilokrat S (2008) Linkage Group Selection to Investigate Genetic Determinants of Complex Traits of Malaria Parasites. PhD Thesis: University of Edinburgh.
Pattaradilokrat S, Cheesman SJ, Carter R (2007) Linkage group selection: towards identifying genes controlling strain specific protective immunity in malaria. PLoS ONE 2(9): e857.

Pattaradilokrat S, Cheesman SJ, Carter R (2008) Congenicity and genetic polymorphism in cloned lines derived from a single isolate of a rodent malaria parasite. Mol Biochem Parasitol 157(2): 244-247.
Perkins SL, Sarkar IN, Carter R (2007) The phylogeny of rodent malaria parasites: simultaneous analysis across three genomes. Infect Genet Evol 7(1): 74-83.
Peterson DS, Wellems TE (2000) EBL-1, a putative erythrocyte binding protein of Plasmodium falciparum, maps within a favored linkage group in two genetic crosses. Mol Biochem Parasitol 105(1): 105-113.
Peterson DS, Walliker D, Wellems TE (1988) Evidence that a point mutation in dihydrofolate reductase-thymidylate synthase confers resistance to pyrimethamine in falciparum malaria. Proc Natl Acad Sci U S A 85(23): 9114-9118.
Pradel G, Frevert U (2001) Malaria sporozoites actively enter and pass through rat Kupffer cells prior to hepatocyte invasion. Hepatology 33(5): 1154-1165.
Prudencio M, Mota MM (2007) To migrate or to invade: those are the options. Cell Host Microbe 2(5): 286-288.
Raberg L, de Roode JC, Bell AS, Stamou P, Gray D et al. (2006) The role of immune-mediated apparent competition in genetically diverse malaria infections. Am Nat 168(1): 41-53.
Ranford-Cartwright LC, Balfe P, Carter R, Walliker D (1993) Frequency of cross-fertilization in the human malaria parasite Plasmodium falciparum. Parasitology 107 (Pt 1): 11-18.
Ranjit MR, Das A, Das BP, Das BN, Dash BP et al. (2005) Distribution of Plasmodium falciparum genotypes in clinically mild and severe malaria cases in Orissa, India. Trans R Soc Trop Med Hyg 99(5): 389-395.
Read A, Mackinnon M, Anwar M, Taylor L (2002) Kin-selection models as evolutionary explanations of Malaria. In: Adaptive dynamics of infectious diseases: In pursuit of virulence management. pp165-178; Dieckmann U, Metz J, Sabelis M, Sigmund K, editors: Cambridge University Press.
Read AF, Taylor LH (2001) The ecology of genetically diverse infections. Science 292(5519): 1099-1102.
Reilly HB, Wang H, Steuter JA, Marx AM, Ferdig MT (2007) Quantitative dissection of clone-specific growth rates in cultured malaria parasites. Int J Parasitol 37(14): 1599-1607.
Ribacke U, Mok BW, Wirta V, Normark J, Lundeberg J et al. (2007) Genome wide gene amplifications and deletions in Plasmodium falciparum. Mol Biochem Parasitol 155(1): 33-44.
Richie TL (1988) Interactions between malaria parasites infecting the same vertebrate host. Parasitology 96 (Pt 3): 607-639.
Robert F, Ntoumi F, Angel G, Candito D, Rogier C et al. (1996) Extensive genetic diversity of Plasmodium falciparum isolates collected from patients with severe malaria in Dakar, Senegal. Trans R Soc Trop Med Hyg 90(6): 704-711.
Ronaghi M, Uhlen M, Nyren P (1998) A sequencing method based on real-time pyrophosphate. Science 281(5375): 363, 365.
Rowe A, Obeiro J, Newbold CI, Marsh K (1995) Plasmodium falciparum rosetting is associated with malaria severity in Kenya. Infect Immun 63(6): 2323-2326.

Rowe JA, Obiero J, Marsh K, Raza A (2002) Short report: Positive correlation between rosetting and parasitemia in Plasmodium falciparum clinical isolates. Am J Trop Med Hyg 66(5): 458-460.
Rowe JA, Handel IG, Thera MA, Deans AM, Lyke KE et al. (2007) Blood group O protects against severe Plasmodium falciparum malaria through the mechanism of reduced rosetting. Proc Natl Acad Sci U S A 104(44): 17471-17476.
Sachs J, Malaney P (2002) The economic and social burden of malaria. Nature 415(6872): 680-685.
Scorza T, Grubb K, Smooker P, Rainczuk A, Proll D et al. (2005) Induction of strain-transcending immunity against Plasmodium chabaudi adami malaria with a multiepitope DNA vaccine. Infect Immun 73(5): 2974-2985.
Simpson JA, Silamut K, Chotivanich K, Pukrittayakamee S, White NJ (1999) Red cell selectivity in malaria: a study of multiple-infected erythrocytes. Trans R Soc Trop Med Hyg 93(2): 165-168.
Simpson JA, Aarons L, Collins WE, Jeffery GM, White NJ (2002) Population dynamics of untreated Plasmodium falciparum malaria within the adult human host during the expansion phase of the infection. Parasitology 124(Pt 3): 247-263.
Smalley ME, Brown J, Bassett NM (1981) The rate of production of Plasmodium falciparum gametocytes during natural infections. Trans R Soc Trop Med Hyg 75(2): 318-319.
Snounou G, White NJ (2004) The co-existence of Plasmodium: sidelights from falciparum and vivax malaria in Thailand. Trends Parasitol 20(7): 333-339.
Snounou G, Jarra W, Viriyakosol S, Wood JC, Brown KN (1989) Use of a DNA probe to analyse the dynamics of infection with rodent malaria parasites confirms that parasite clearance during crisis is predominantly strain- and species-specific. Mol Biochem Parasitol 37(1): 37-46.
Snounou G, Bourne T, Jarra W, Viriyakosol S, Wood JC et al. (1992) Assessment of parasite population dynamics in mixed infections of rodent plasmodia. Parasitology 105 (Pt 3): 363-374.
Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI (2005) The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434(7030): 214-217.
Snow RW, Schellenberg JR, Peshu N, Forster D, Newton CR et al. (1993) Periodicity and space-time clustering of severe childhood malaria on the coast of Kenya. Trans R Soc Trop Med Hyg 87(4): 386-390.
Su X, Hayton K, Wellems TE (2007) Genetic linkage and association analyses for trait mapping in Plasmodium falciparum. Nat Rev Genet 8(7): 497-506.
Su X, Kirkman LA, Fujioka H, Wellems TE (1997) Complex polymorphisms in an approximately 330 kDa protein are linked to chloroquine-resistant P. falciparum in Southeast Asia and Africa. Cell 91(5): 593-603.
Su X, Ferdig MT, Huang Y, Huynh CQ, Liu A et al. (1999) A genetic map and recombination parameters of the human malaria parasite Plasmodium falciparum. Science 286(5443): 1351-1353.
Taylor LH, Read AF (1998) Determinants of transmission success of individual clones from mixed-clone infections of the rodent malaria parasite, Plasmodium chabaudi. Int J Parasitol 28(5): 719-725.

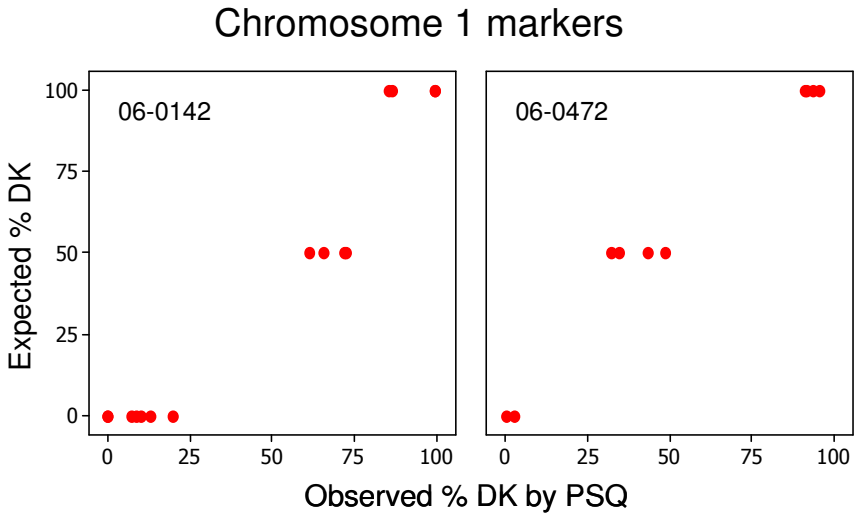
Taylor LH, Walliker D, Read AF (1997) Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. Proc Biol Sci 264(1383): 927-935.
Taylor LH, Mackinnon MJ, Read AF (1998) Virulence of mixed-clone and single-clone infections of the rodent malaria Plasmodium chabaudi. Evolution 52(2): 583-591.
Tewari R, Ogun SA, Gunaratne RS, Crisanti A, Holder AA (2005) Disruption of Plasmodium berghei merozoite surface protein 7 gene modulates parasite growth in vivo. Blood 105(1): 394-396.
Timms R, Colegrave N, Chan BH, Read AF (2001) The effect of parasite dose on disease severity in the rodent malaria Plasmodium chabaudi. Parasitology 123(Pt 1): 1-11.
Toure FS, Ouwe-Missi-Oukem-Boyer O, Mezui-Me-Ndong J, Ndong-Atome GR, Bisvigou U et al. (2007) Cytoadherence and Genotype of Plasmodium falciparum Strains from Symptomatic Children in Franceville, Southeastern Gabon. Clin Med Res 5(2): 106-113.
Trembley HL, Greenberg J (1954) Further studies on the hybridization of strains of Plasmodium gallinaceum. J Parasitol 40(4): 475-479.
Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23(21): 4407-4414.
Voza T, Vigario AM, Belnoue E, Gruner AC, Deschemin JC et al. (2005) Species-specific inhibition of cerebral malaria in mice coinfected with Plasmodium spp. Infect Immun 73(8): 4777-4786.
Walker-Jonah A, Dolan SA, Gwadz RW, Panton LJ, Wellems TE (1992) An RFLP map of the Plasmodium falciparum genome, recombination rates and favored linkage groups in a genetic cross. Mol Biochem Parasitol 51(2): 313-320.
Walliker D (2005) The hitchhiker's guide to malaria parasite genes. Trends Parasitol 21(11): 489-493.
Walliker D, Carter R, Morgan S (1971) Genetic recombination in malaria parasites. Nature 232(5312): 561-562.
Walliker D, Carter R, Sanderson A (1975) Genetic studies on Plasmodium chabaudi: recombination between enzyme markers. Parasitology 70(1): 19-24.
Walliker D, Babiker HA, &, Ranford-Cartwright LC (1998) Chapter 16 in Malaria: Parasite Biology, Pathogenesis and Protection; Sherman IW, editor. Washington, D.C: ASM press.
Walliker D, Sanderson A, Yoeli M, Hargreaves BJ (1976) A genetic investigation of virulence in a rodent malaria parasite. Parasitology 72(2): 183-194.
Walliker D, Quakyi IA, Wellems TE, McCutchan TF, Szarfman A et al. (1987) Genetic analysis of the human malaria parasite Plasmodium falciparum. Science 236(4809): 1661-1666.
Wargo AR, de Roode JC, Huijben S, Drew DR, Read AF (2007) Transmission stage investment of malaria parasites in response to in-host competition. Proc Biol Sci 274(1625): 2759-2768.
Warrell D, Molyneux, ME and Beales PF. (1990) Severe and uncomplicated malaria, 2nd edition. Trans R Soc Trop Med Hyg 84 supplement 2.
Wellems TE, Walker-Jonah A, Panton LJ (1991) Genetic mapping of the chloroquine-resistance locus on Plasmodium falciparum chromosome 7. Proc Natl Acad Sci U S A 88(8): 3382-3386.

Wellems TE, Panton LJ, Gluzman IY, do Rosario VE, Gwadz RW et al. (1990) Chloroquine resistance not linked to mdr-like genes in a Plasmodium falciparum cross. Nature 345(6272): 253-255.
Wellems TE, Walliker D, Smith CL, do Rosario VE, Maloy WL et al. (1987) A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of Plasmodium falciparum. Cell 49(5): 633-642.
White NJ (1998) Chapter 26: Malaria Pathophysiology in Malaria: Parasite Biology, Pathogenesis and Protection; Sherman IW, editor. Washington, DC: ASM Press.
WHO (2000) World Health Organisation expert committee on Malaria, 20th Report. Geneva.
WHO (2001) World Health Organisation Roll Back Malaria partnership. Geneva.
Wirth DF (2002) Biological revelations. Nature 419(6906): 495-496.
Wootton JC, Feng X, Ferdig MT, Cooper RA, Mu J et al. (2002) Genetic diversity and chloroquine selective sweeps in Plasmodium falciparum. Nature 418(6895): 320-323.
Xu H, Hodder AN, Yan H, Crewther PE, Anders RF et al. (2000) CD4+ T cells acting independently of antibody contribute to protective immunity to Plasmodium chabaudi infection after apical membrane antigen 1 immunization. J Immunol 165(1): 389-396.

Appendix I : Pyrosequencing Markers

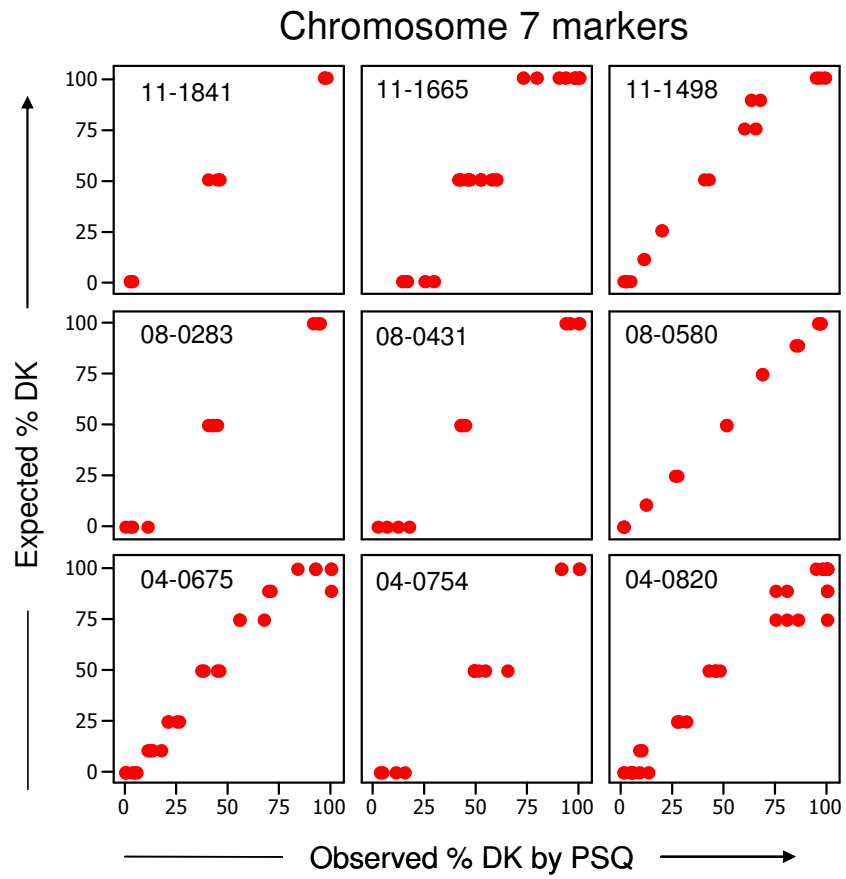
Pyrosequencing SNP markers were designed on chromosome 1 (Figure I.a), chromosome 7 (Figure I. b) and chromosome 9 (Figure I.c). The location of each marker is given, along with the Spearman's rank correlation for each marker for the quantification by pyrosequencing of the proportions of DK in control mixtures of DS and DK.

Figure I.a
Two markers on chromosome 1 tested for correlations between the expected proportion of DK in 3 controlled mixtures and that measured by pyrosequencing.



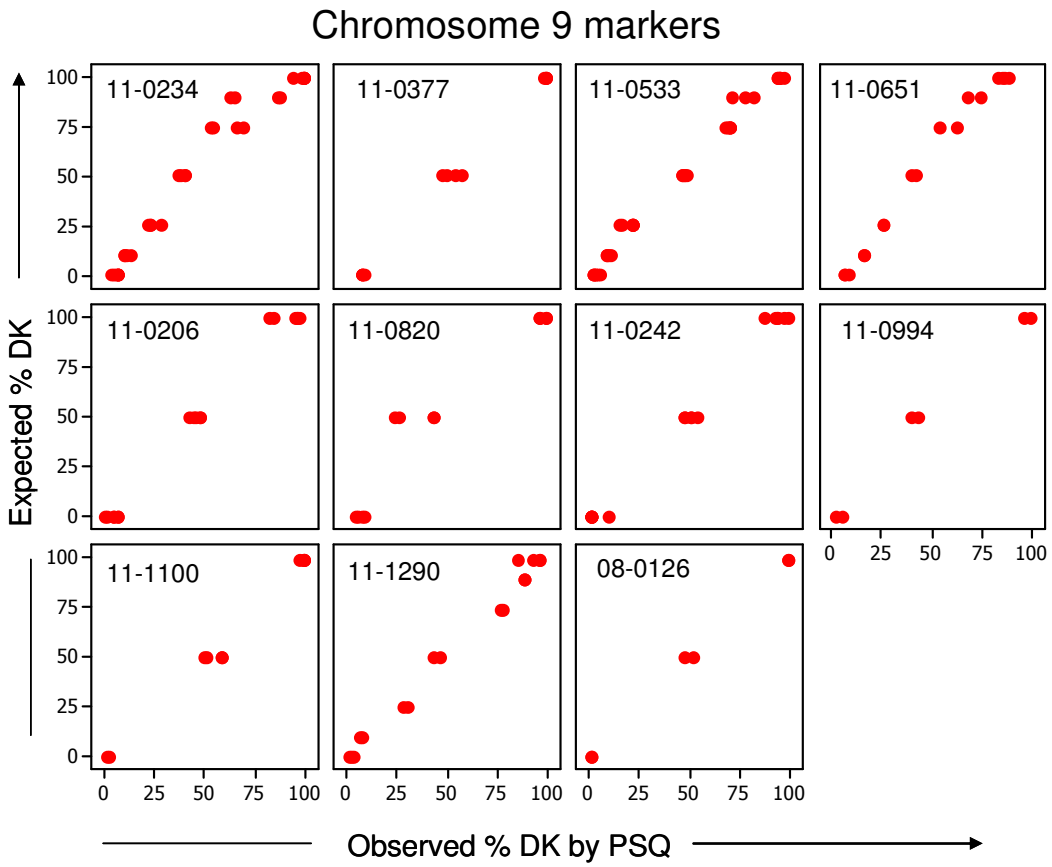
Marker Number	Marker Name	*P.chabaudi* gene	*P.falciparum* orthologous gene	Location on chromosome (kb)	Spearman's Rank correlation with mixture controls r_s (p value)
1	06-0142	PC000378.05.0	MAL6P1.24	9.9	0.931 (p<0.001)
2	06-0472	PC000444.03.0	MAL6P1.101	339	0.934 (p<0.001)

Figure I.b
Nine markers on chromosome 7 tested for correlations between the expected proportion of DK in 3 controlled mixtures (5 markers) or in 7 controlled mixtures (4 markers) and that measured by pyrosequencing.



Marker Number	Marker Name	*P.chabaudi* gene	*P.falciparum* orthologous gene	Location on chromosome (kb)	Spearman's Rank correlation with mixture controls r_s (p value)
1	11-1841	PC301222.00.0	PF11_0479	70	0.958 (p<0.001)
2	11-1665	PC000657.02.0	PF11_0427	245	0.932 (p<0.001)
3	11-1498	PC000330.03.0	PF11_0395	412	0.982 (p<0.001)
4	08-0283	PC000059.04.0	MAL8P1.32	565	0.946 (p<0.001)
5	08-0431	PC000663.02.0	MAL8P1.58	712	0.949 (p<0.001)
6	08-0580	PC000572.02.0	MAL8P1.78	864	0.993 (p<0.001)
7	04-0675	PC000665.03.0	PFD0725c	952	0.982 (p<0.001)
8	04-0754	PC000104.03.0	PFD0805w	1010	0.944 (p<0.001)
9	04-0820	PC000130.01.0	PFD0875c	1094	0.940 (p<0.001)

Figure I.c
Eleven markers on chromosome 9 tested for correlations between the expected proportion of DK in 3 controlled mixtures (7 markers) or in 7 controlled mixtures (4 markers) and that measured by pyrosequencing.



Marker Number	Marker Name	*P.chabaudi* gene	*P.falciparum* orthologous gene	Location on chromosome (kb)	Spearman's Rank correlation with mixture controls r_s (p value)
1	11-0234	PC000415.04.0	PF11_0065	96	0.983 (p<0.001)
2	11-0377	PC000630.04.0	PF11_0101	239	0.954 (p<0.001)
3	11-0533	PC001036.02.0	PF11_0147	395	0.988 (p<0.001)
4	11-0651	PC000213.02.0	PF11_0177	513	0.990 (p<0.001)
5	11-0206	PC000554.02.0	PF11_0206	608	0.944 (p<0.001)
6	11-0820	PC000771.03.0	PF11_0225	680	0.948 (p<0.001)
7	11-0242	PC000595.02.0	PF11_0242	768	0.955 (p<0.001)
8	11-0994	PC000324.04.0	PF11_0265	857	0.956 (p=0.003)
9	11-1100	PC000814.00.0	PF11_0294	957	0.949 (p<0.001)
10	11-1290	PC301665.00.0	PF11_0344	1153	0.973 (p<0.001)
11	08-0126	PC000686.03.0	PF08_0010	1324	0.985 (p<0.001)

Appendix II: Clonal Cross Progeny Marker Inheritance

From the data in Chapters 5 and 6, it was clear that a large region (around 400kb in length) at the left hand side of chromosome 9 was consistently, strongly selected against in all the fast-growing crosses and backcrosses examined. This raised the possibility that either; (1) more than one locus in this region determined growth and these loci must be inherited together to give the fast growth phenotype, or (2) only one locus was important for fast growth but the whole region was inherited as either DS or DK due to a block to recombination within the region. In order to try and distinguish between these two possibilities, an attempt was made to clone out the unselected cross C progeny (from the initial sporozoite-induced infection) and to look for the presence of recombinants in the region of interest. The rationale was that if recombinants in this region had arisen by crossing, and were found in the unselected cross progeny, there must not be a block to recombination between DS and DK. If this was the case, it would mean that when only cross progeny inheriting DS right across this region are found after selection, the whole region must be required for fast growth.

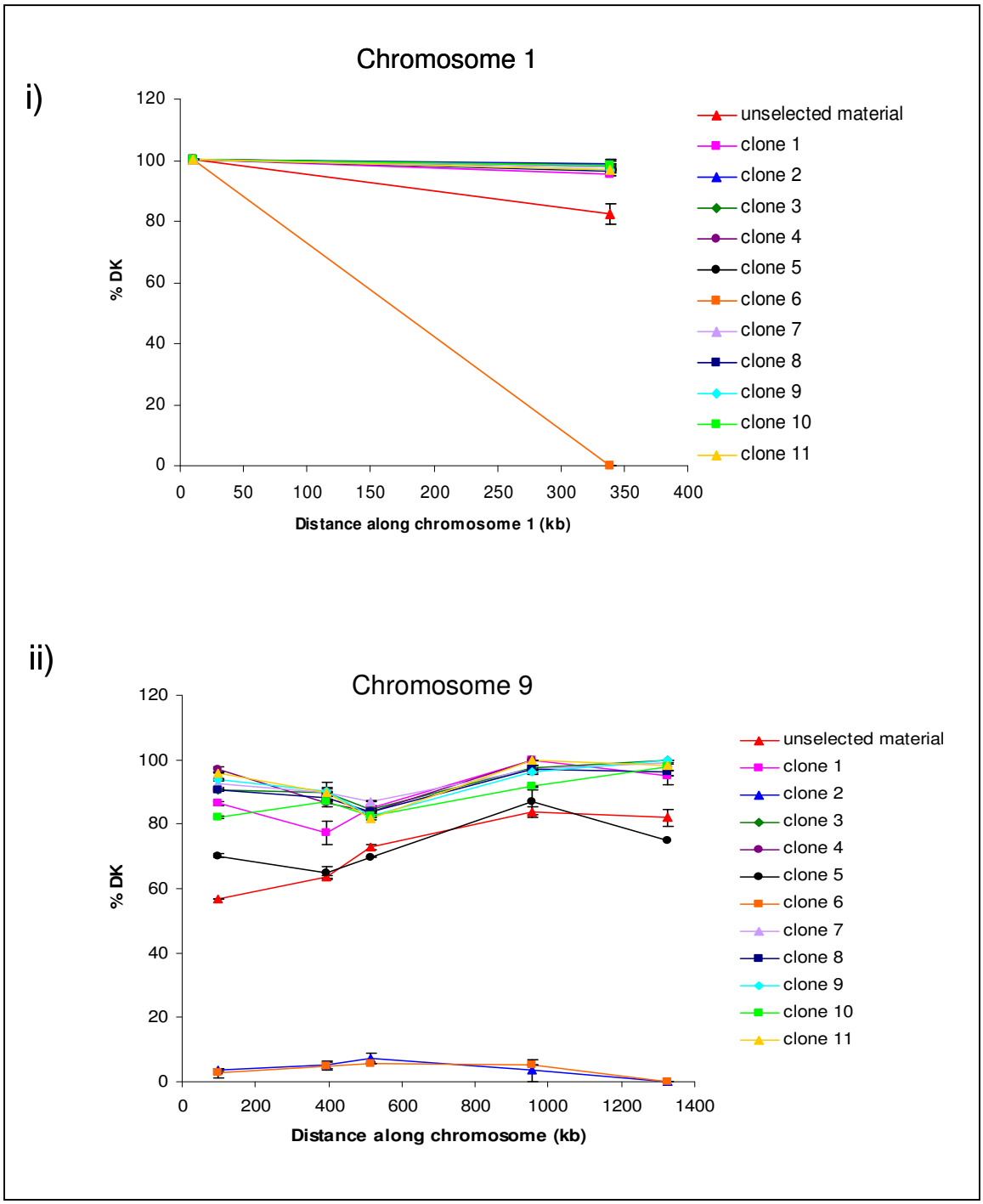
The blood of the two mice with the sporozoite-induced infections of cross C progeny was pooled at day 4 of infection as described in Chapter 5. This was frozen as a single stabilate in liquid nitrogen (unselected cross C progeny). It was removed and inoculated into a single CBA female mouse, allowed to grow to 0.59% over 6 days and then sub-inoculated into groups of C57 mice for cloning. At this point, a blood sample was taken from the infection of the unselected cross progeny pool, to assess

DK marker inheritance on chromosome 9 and to check that selection had not already begun. At the same time, cross C progeny were diluted to a range of inoculum sizes: 1, 3, 10, 30 or 100 parasites/inoculum, and inoculated into a total of 75 mice. Groups of mice were infected with different inoculum sizes because it was unclear how much the viability of the parasites would decrease before inoculation and 1 parasite per mouse was required for successful cloning. Volumes of donor mouse blood were diluted in 50% Fetal Calf Serum 50% Ringers solution, made up to 1% haematocrit by adding uninfected mouse blood, and then inoculated into recipient mice. One group of 5 mice were infected with an estimated 1 pRBC/mouse, one group of 30 mice with 3 pRBC/mouse, one group of 30 mice with 10 pRBC/mouse, one group of 5 mice with 30 pRBC/mouse and a final group of 5 mice with 100 pRBC/mouse, to give 75 mice in total.

Blood samples were taken for marker inheritance quantification from mice that became positive for infection. Of the 75 mice infected, only 11 developed an infection. These were 0/5 mice given 1 pRBC, 1/30 mice given 3 pRBC, 5/30 mice given 10 pRBC, 2/5 mice given 30 pRBC and 3/5 mice given 100 pRBC. Therefore, it was clear that parasite viability had decreased severely and actual parasite inocula were much smaller than calculated.

The inheritance of 5 DK markers on chromosome 9 was measured for the 11 putative clones (described here simply as “clones”) and the unselected, uncloned material (Figure II.a). It was clear that strong selection had not occurred on the material used for cloning, except for the first DK marker on chromosome 9 which was already

Figure II.a
The inheritance of DK markers on chromosome 1 (i) and chromosome 9 (ii) by putative clones isolated from the unselected cross C progeny pool, and by the unselected cross C progeny pool itself. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.



starting to decrease slightly. One of the clones (clone 5) appeared to have the same marker inheritance pattern as the unselected material, indicating it had not been successfully cloned and was still a mixture. Eight of the remaining clones appeared to inherit high levels of DK right across chromosome 9, whereas 2 clones inherited very little DK. This can be interpreted as 10 clones which were parental-type – 8 DK and 2 DS, across chromosome 9. Therefore no recombination events were found at any point across chromosome 9 in this small number of parasites. By checking DK inheritance on chromosome 1 it was clear that one of the clones (clone 6) with DS parental-type chromosome 9, actually had a recombination event on chromosome 1 between markers 1 and 2 (Figure II.a.i). Another (clone 2) had a DS chromosome 9 and a DK chromosome 1 (Figure II.a.i). However, of the only 2 proven recombinants (clones 2 and 6), there were no recombination events in the region of interest on chromosome 9, as the three markers tested were all of the DS type in this region.

Therefore, this attempt at cloning was not successful in producing a sufficient number of cross progeny clones for the purpose of testing whether recombination could, or could not, occur readily in the region of interest on chromosome 9. Due to time considerations, cloning was not attempted again, however, in the future this approach should be repeated. Demonstrating whether or not there is a block to recombination in the region of interest would lead to an understanding of how many candidate loci it might contain.

Another strategy involving cloning, which would be of use in the future, is to clone out fully selected fast-growing cross progeny, and type them for marker inheritance

across the region of interest. More markers would need to be generated in the region of interest on chromosome 9, and many recombinant clones would need to be isolated but this could narrow down the region (Figure II. b). Cloning was accidentally achieved in this way on one occasion, during the creation and selection of an additional cross, cross F.

Cross F was generated, at the same time as crosses D and E, from a mixed infection of two mice with a 1:4 ratio of DS:DK, which were fed on the same cage of around 250 female mosquitoes on day 6 of infection. On day 8 of the mosquito infection, 6 infected mosquito guts (with oocyst burdens ranging from 14 to >100) were used to determine the number of recombinants. One marker on each of chromosomes 1, 7 and 9 was measured for the proportion of DK, these gave averages of: 95.4% DK / 4.6% DS (from a mixed infection initiated with 80% DK / 20% DS) and the estimated total number of recombinants was 1768 (Figure II.c.i).

Sporozoite-induced infections were generated in a single mouse and 10^6 pRBCs were passaged on into one mouse for the 1st round of fast growth selection on day 9 at 0.43% parasitaemia (Figure II.c.ii). For the 2nd round of selection, 10^6 pRBCs were passaged on into a single mouse on day 8. Cross F was then frozen down as stabilates in liquid nitrogen on day 8 of infection. For the 3rd round of selection, a stabilate was removed from storage and inoculated into one mouse and grown for 9 days before again being stored in liquid nitrogen. Finally, for the 4th round of selection, a stabilate was removed from storage and inoculated into one mouse and grown for 8 days, before a final blood sample was taken. The progeny of cross F had a sigmoid

Figure II.b
Diagram of cloning strategy to narrow down the region under selection on chromosome 9, by typing many fast-growing cross progeny clones for the inheritance of several DK/DS markers in the region of interest on chromosome 9. Analysis of commonly inherited DS markers between clones could narrow the region down, e.g. from the outer to the inner pair of dashed lines.

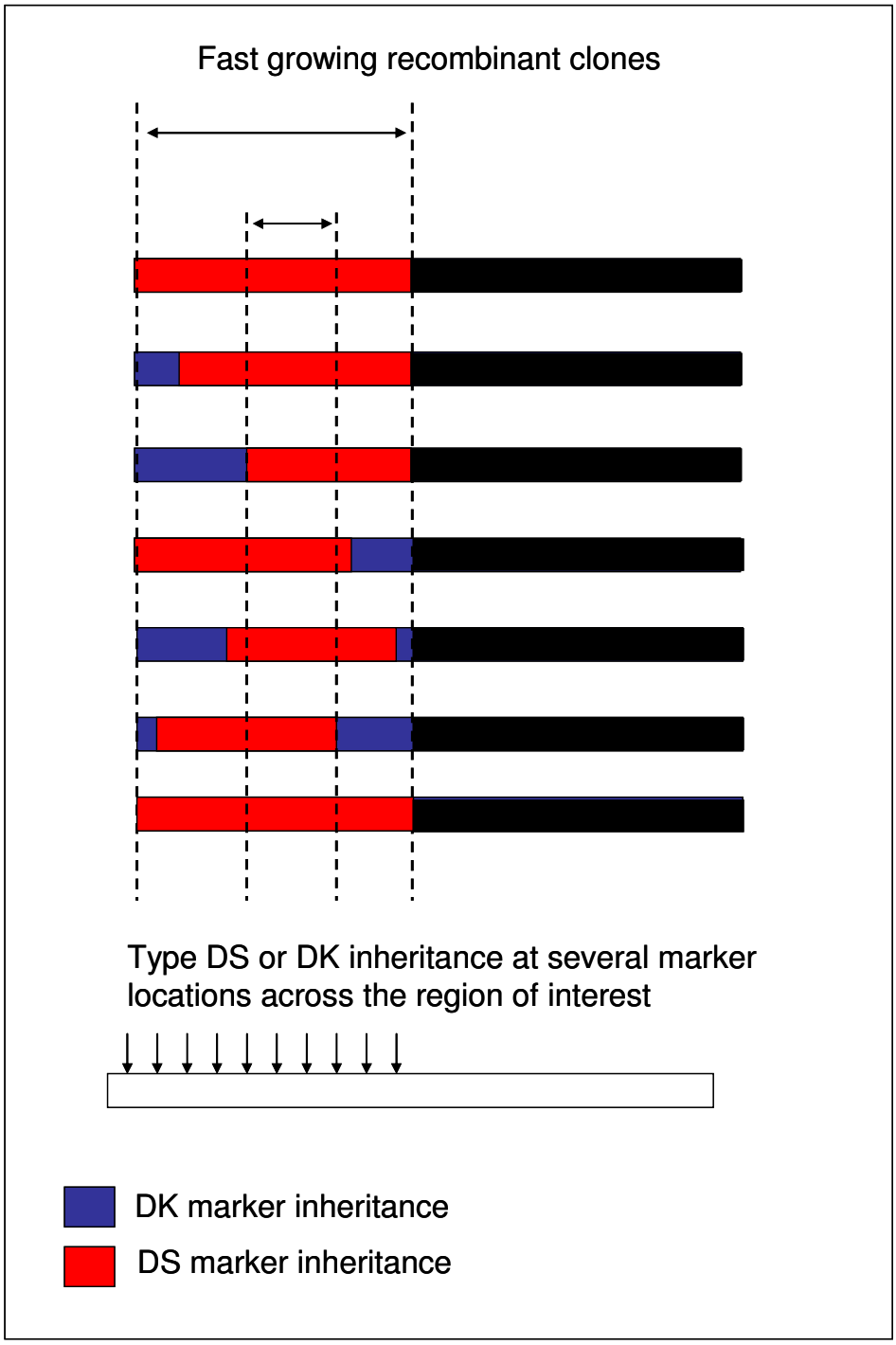
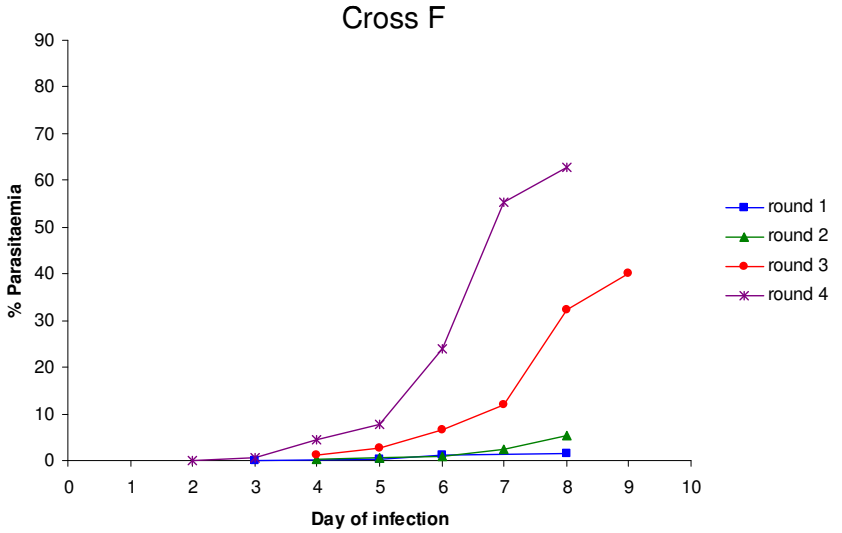


Figure II.c
(i) The transmission success and numbers of recombinants in cross F. Cross F was generated by mosquitoes taking a blood meal on day 6 of infection from two mice which were initially infected with a DS:DK mixture of 1:4. (ii) Selection of cross F progeny for fast growth over four rounds of blood passage. Each line represents the parasitaemia of an individual mouse. 10^6 pRBCs were taken for passage for the 1st and 2nd rounds of selection. For the 3rd and 4th rounds of selection, cross progeny stabilates, from the 2nd and 3rd rounds of selection respectively, were removed from deep freeze storage and inoculated directly into mice.

i)

Cross progeny name	% Mosquitoes infected (mean oocysts / infected gut)	Number of mosquitoes dissected	Average dissected mosquito burden	Number of meiotic products (proportion recombinants)	Theoretical maximum # recombinants
Cross F	75 (54)	124	5022	20088 (0.088)	1768

ii)



growth profile and were relatively slow-growing until the 4th round of selection, when 60% parasitaemia was reached by day 8 (Figure II.c.iii).

However, the DK marker inheritance patterns across chromosomes 1, 7 and 9 indicated that there may only have been a single surviving recombinant clone present after full growth selection, despite starting with an estimate of over 1000 (Figure II.d). However, it is not known whether this number of recombinants successfully entered the liver. If sporozoite viability was for some reason very poor, few might have been inoculated into the mouse. Markers were either inherited at around 30% or around 100% DK suggesting also the presence of a DK parental parasite, since values were not 0% and 100% DK, as would be expected for a single clone infection.

It was clear that one recombination event between DS and DK had taken place on chromosome 7, and two on chromosome 9. Therefore, this fast-growing clone had inherited DS at marker positions 1-4 on chromosome 7 and marker positions 1, 2, 3, 10 and 11 on chromosome 9. However, many more fast-growing recombinant clones would need to be examined in order to draw any further conclusions about the number and location of loci controlling the fast-growth phenotype on chromosomes 7 and 9. But this approach might be valuable in elucidating which parts of chromosome 7 can be inherited in an "and/or" fashion and still be associated with fast growth, as hypothesised based on the marker inheritances of pooled cross progeny in Chapter 5.

Figure II.d
The proportion of the cross F progeny inheriting DK at (i) 2 marker positions on chromosome 1, (ii) 9 marker positions on chromosome 7 and (iii) 10 marker positions on chromosome 9, after the 4th round of selection for fast growth. Means of replicate measurements at the same marker are given, and error bars represent the standard error of the mean.